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EDITED BY

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F. DUNHAM, New York City      A. N. RICHARDS, Philadelphia, Pa.

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# ON THE EXTRACTION OF A SUBSTANCE FROM THE SPERM OF A SEA-URCHIN (*STRONGYLOCENTROTUS PURPURATUS*) WHICH WILL FERTILIZE THE EGGS OF THAT SPECIES

By T. BRAILSFORD ROBERTSON

(From the Rudolph Sprckels Physiological Laboratory of the University of California )

(Received for publication, March 14, 1912 )

From time to time various unsuccessful attempts have been made to extract a substance from sperm which will fertilize eggs of the same species. All of these attempts, as Loeb has pointed out,<sup>1</sup> have either been unsuccessful or else open to the criticism that live sperm were present or that the sea water in which the extract was dissolved was hypertonic or hyperalkaline and thus brought about artificial parthenogenesis.

I have recently succeeded in extracting a substance from the sperm of *Strongylocentrotus purpuratus*, which is capable of fertilizing the eggs of that species, by a method which is not open to these criticisms. In my first experiment I proceeded as follows:

Eighteen testes from mature males of *Strongylocentrotus purpuratus* were immersed in 65 cc of distilled water and allowed to stand in ice for two or three days. To this mixture were then added 165 cc of distilled water. The total volume of the mixture was now 340 cc. To this mixture were added a few cubic centimeters of ether in order to complete the cytolysis of the cells and the mixture was allowed to stand at room temperature for about one hour, being thoroughly shaken from time to time by hand. To this were then added 40 cc of  $\frac{5M}{2}$  NaCl, thus rendering it isotonic with sea water, and the mixture was then shaken very thoroughly.

<sup>1</sup> J. Loeb, *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909.

and allowed to stand at room temperature over night. The following morning the mixture was again shaken and then very thoroughly centrifuged until a perfectly clear fluid layer was obtained, floating above the residue. This clear, brownish fluid, upon which were floating a few fragments of connective tissue, was then filtered. Filtration was very rapid and a clear, brownish, very slightly opalescent filtrate was obtained of which the volume was 200 cc. To this were added 600 cc of acetone. A copious flocculent precipitate resulted which was collected on a hardened filter, washed in 500 cc of alcohol twice, and then twice in 500 cc of ether<sup>2</sup> and then left to dry over  $H_2SO_4$  in an incubator at  $36^\circ$  for twelve hours.

The product was a pale violet, lightly caked powder. It was pulverized and sifted through a very fine sieve and then dried further over  $H_2SO_4$  at  $37^\circ$  for three days. About 1 gram of material was obtained.

One-quarter gram of this material was rubbed up to a paste with a little filtered sea water and the volume was then made up to 50 cc with sea water and the mixture thoroughly stirred and allowed to stand at room temperature for one hour. A considerable proportion of insoluble residue which was purple in color remained, this was centrifuged off and a clear, pale yellowish, slightly opalescent fluid was obtained. This fluid was diluted to one, one-half, one-fourth and one-sixteenth with sea water.

To 2 cc of each of these mixtures were added 2 drops (A) of the ripe eggs of *Strongylocentrotus purpuratus* which had not undergone previous treatment of any kind save that of washing in sea water or (B) of the eggs of the same female which had been previously treated by four minutes' immersion in  $\frac{M}{2}$   $SrCl_2$ . The following were the results obtained.

- These washings were conducted within the incubator over  $H_2SO_4$  in order to avoid the deposition of atmospheric moisture upon the filter. The same remark also applies to the preparations described below.

DILUTION OF THE ORIGINAL SOLUTION OF SPERM EXTRACT	EFFECT ON EGGS NOT PREVIOUSLY TREATED	EFFECT ON EGGS PREVIOUSLY TREATED WITH $\text{SrCl}_2$
1	Distinct spherical membranes on a number in fifteen minutes In forty minutes 100 per cent have formed membranes	In twenty minutes distinct spherical membranes on 100 per cent
$\frac{1}{2}$	In forty-five minutes no membranes had formed	In seven minutes membranes beginning in several In thirty minutes 30 per cent have membranes In 40 minutes, 44 per cent
$\frac{1}{4}$	In forty-five minutes no effects	In thirty minutes about 30 per cent have perfect spherical membranes
$\frac{1}{8}$	In forty-five minutes no effect	In thirty minutes several have perfect membranes
$\frac{1}{16}$	In forty-five minutes no effect	In fifteen minutes blisters or thin membranes on many of the eggs

A repetition of the experiment yielded identical results

From these results I was at first inclined to conclude that I had succeeded in extracting the fertilizing agent from sperm. My colleague, Dr A R Moore, however, who repeated these experiments<sup>3</sup> and confirmed them, pointed out to me that the membranes, at any rate those which were formed by unsensitized eggs, were certainly not true fertilization membranes, for they were permeable to sperm and, after a considerable delay, the true fertilization membrane was formed by sperm underneath them. They appeared, therefore, to be formed by a modification of the zona pellucida of such a nature as to render it readily visible, and to be analogous to the "Pseudo-membrane" described by Loeb<sup>4</sup>. This conclusion received further support from the fact that the eggs upon which these pseudo-membranes had been formed did not undergo cleavage upon standing.

From numerous experiments upon the fertilization of eggs by

<sup>3</sup> Employing sperm, not spermaries

<sup>4</sup> J Loeb *Arch f d ges Physiol*, cxxiv, p 48, 1908



sera I have gathered the impression that the formation of pseudo-membrane is a process closely connected with that of true membrane-formation<sup>5</sup> Moreover these extracts agglutinated the eggs and I have found that agglutination frequently accompanies fertilization It accordingly appeared to me quite possible that sperm extract prepared in the above manner contained a trace of the fertilizing agent but that some detail in the method of extraction was responsible for its incompleteness

In seeking for a reason for my failure to completely extract the fertilizing agent from sperm it occurred to me that the fertilizing agent in blood-sera is precipitated or at any rate rendered sparingly soluble by calcium salts<sup>6</sup> If, therefore, as seems very probable, the fertilizing agent in sperm is identical with that which is found in blood-sera, the presence of calcium salts in the fluid employed to extract the fertilizing agent might be expected to hinder or even altogether prevent the extraction Now the sperm, in the experiments described above and in Dr Moore's experiments, were suspended in sea-water and distilled water was added to this suspension in order to cytolyze them, consequently the fluid employed to extract the fertilizing agent contained calcium salts derived from sea-water It therefore appeared possible that the associated sea-water was responsible for the failure to completely extract the fertilizing agent This deduction I was able to confirm in the following manner

A The sperm of sixteen *purpuratus* males, thickly suspended in sea-water, were dropped into 100 cc of distilled water to which had been added a few cubic centimeters of ether This mixture stood on ice for two days and at room temperature for some hours, being shaken from time to time The total volume of the mixture was 190 cc To this were then added 18 cc of  $\frac{1}{2}$  NaCl and a few more cubic centimeters of ether The mixture was shaken well and allowed to stand at room temperature for twelve hours The mixture was then centrifuged and the supernatant fluid filtered The volume of the filtrate was 128 cc To this were added 64 cc of 7 per cent BaCl<sub>2</sub> and the mixture was centrifuged A copious membranous white precipitate settled to the bottom of the centrifuge tubes This precipitate was drained thoroughly, then suspended in 200 cc of 2 per cent BaCl<sub>2</sub> and centrifuged again

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<sup>5</sup> Thus a sample of undiluted blood serum, isotonic with sea-water, which failed to fertilize sea-urchin eggs will, nevertheless, after one to two hours, frequently cause the appearance of "pseudo-membrane" upon the eggs

<sup>6</sup> Cf T Brailsford Robertson *This Journal*, 21, p 339, 1912

The precipitate, after draining thoroughly, was stirred up rapidly in 100 cc of  $\frac{N}{10}$  HCl for one hour. Little or none of the precipitate appeared to dissolve. To this mixture was added 5 cc of 10 per cent  $\text{Na}_2\text{SO}_4$ , the mixture was then centrifuged and the fluid filtered. To the filtrate, which contained no barium, were added 400 cc of acetone, *no precipitate resulted, the mixture remaining perfectly clear*. On standing overnight a very small trace of a flocculent precipitate settled out, which was too small to collect.

*From this experiment we may conclude, therefore, that sea-water, even when considerably diluted with distilled water does not extract from sperm any appreciable quantity of a substance precipitable by barium, soluble in  $\frac{N}{10}$  HCl and precipitable from this solution by four volumes of acetone*

B The spermaries of twenty-four purpuratus males were cut up and suspended in 1100 cc of sea-water. The thick suspension of sperm thus obtained was filtered through cheese-cloth and then centrifuged until the sperm collected in the bottom of the centrifuge tubes in the form of a thick cake which could be drained thoroughly without loss of sperm. These were then suspended in 400 cc of  $\frac{M}{2}$  NaCl and left upon ice overnight. The following morning this mixture was centrifuged and the supernatant fluid was drained off. The sperm were then again suspended in 400 cc of  $\frac{M}{2}$  NaCl, centrifuged and drained. They were then suspended in 400 cc of distilled water containing 5 cc of ether, shaken very thoroughly and left to stand at room temperature overnight.

The following day I added to this mixture 100 cc of  $\frac{5M}{2}$  NaCl. The final volume of the mixture was 625 cc. I added 5 cc more of ether, shook well, and allowed the mixture to stand at room temperature for half an hour. The mixture was then centrifuged and the supernatant fluid filtered. The very opalescent filtrate measured 390 cc. To this I added 200 cc of 7 per cent  $\text{BaCl}_2$ . No precipitate resulted, but the opalescence of the mixture was markedly increased. I then placed the mixture in an incubator and heated it to  $50^\circ$  to  $55^\circ\text{C}$  for one and one-half hours at the end of which time a copious flocculent precipitate had formed. The mixture was now allowed to stand on ice overnight.

The following day the mixture was centrifuged. The precipitate was suspended in 200 cc of 2 per cent BaCl and again centrifuged. This washing was repeated. The mixture was then suspended in 100 cc of  $\frac{N}{10}$  HCl. The greater part of the precipitate appeared to be coagulated or curdled by the acid. The mixture was stirred for one hour, 10 cc of 10 per cent  $\text{Na}_2\text{SO}_4$  were added, the mixture was allowed to stand on ice overnight, and then filtered. To the clear white filtrate which was freed from barium, I added four volumes of acetone. *A very considerable flocculent white precipitate at once came down*. This precipitate was collected on a hardened filter, washed in 500 cc of alcohol in several portions, and in 100 cc of ether and dried over  $\text{H}_2\text{SO}_4$  at  $37^\circ\text{C}$  overnight.

The residual portion of the barium precipitate which was insoluble in dilute acid, was very considerable in amount. Without washing, I allowed it to drain thoroughly and then scraped it off the filter-paper, suspended it in 100 cc of  $\frac{N}{10}$  KOH, and shook the mixture thoroughly. After allowing the mixture to stand for three hours I centrifuged it and filtered the supernatant fluid. Only a very small residue remained, obviously  $\text{Ba}_2\text{SO}_4$ , and the filtrate was opalescent and yellowish in color. To this I added four volumes of acetone. A heavy, yellowish, coagulum-like precipitate formed at once, which rolled up into one lump on gently shaking the vessel, like gluten or freshly-precipitated rubber. This was collected upon a hardened filter, washed with alcohol and ether and dried over  $\text{H}_2\text{SO}_4$  at  $37^\circ$  overnight.

*From this experiment we may conclude, therefore, that dilute NaCl solution which is not contaminated by sea-water will extract from sperm two substances which are precipitable by  $\text{BaCl}_2$  and by acetone, the one being soluble in  $\frac{N}{10}$  HCl, the other insoluble in  $\frac{N}{10}$  HCl but soluble in  $\frac{N}{10}$  KOH.*

The following day the acid-soluble substance was found to have dried in friable white flakes which were readily pulverized, 190 mgms of this substance being obtained. The alkali-soluble substance had dried in a horny, brownish cake which was pulverized with difficulty, this product weighed 320 mgms.

After thorough pulverization the powders were spread out in thin layers over  $\text{H}_2\text{SO}_4$  at  $37^\circ$  and dried for a week. I have repeatedly found that dry pulverized proteins, prepared by washing in alcohol and ether, and spread out in thin layers over  $\text{H}_2\text{SO}_4$  at  $37^\circ$  lose weight slightly for twenty-four hours and after forty-eight hours contain no trace of ether or alcohol. The products were therefore certainly free from these reagents. Careful flame-tests, both before and after moistening with nitric acid, failed to reveal the presence of the slightest trace of barium in either of them.

Thirty-eight milligrams of each of these substances were dissolved, the acid-soluble substance in 6.5 cc of  $\frac{N}{5}$  HCl, and the alkali-soluble substance in 6.5 cc of NaOH. The acid-soluble substance dissolved readily and completely, the alkali-soluble substance slowly and incompletely. Both solutions were neutralized, the one by the addition of 6.5 cc of  $\frac{N}{5}$  NaOH and the other by the addition of 6.5 cc of  $\frac{N}{5}$  HCl. Both solutions, after neutralization, were somewhat opalescent. Both solutions were rendered isotonic with sea-water by the addition of 2 cc of  $\frac{5M}{2}$  NaCl and were then diluted to one, one-half, one-fourth, and so forth, by successive additions of filtered sea-water, forming solutions containing one part of the substance to 400, 800, 1600, and so forth, of solvent.

*The acid-soluble substance*

PROPORTION OF SUBSTANCE IN THE MIXTURE	EFFECT ON EGGS NOT PRE- VIOUSLY TREATED	EFFECT ON EGGS PREVIOUSLY TREATED WITH $\text{SrCl}_2$
1:400	Eggs agglutinated* within one minute. After two hours clear hyaline "pseudo-membrane" with very indistinct outlines surrounded each egg. These were at once penetrated by sperm but the underlying eggs were not fertilizable by sperm.	Eggs agglutinated* within one minute. Blisters formed on a number in twenty minutes. 100 per cent distinct but very narrow membranes in two hours.
1:800	Eggs agglutinated. No other effect in two hours.	Eggs agglutinated. In forty minutes irregular cloudy membranes on 50 per cent and cytolysis proceeding in these. In two hours 100 per cent membranes and a few cytolyzed.
1:1600	Slight agglutination. No other effect in two hours.	Eggs agglutinated. In twenty minutes discrete blisters upon the surfaces of some of the eggs. In thirty minutes some have large blisters and many have distinct membranes. In fifty minutes 50 per cent have complete membranes and cytolysis is indicated by granular matter contained in all of these membranes. In one hour 100 per cent have membranes, many of them being clear and hyaline, others cloudy. In two hours a few per cent have been cytolyzed into "shadows."
1:3200	No effect after two hours. Then treated with sperm the eggs formed fertilization membranes after a very noticeable delay (four to five minutes).	Very slight agglutination. In forty minutes 50 per cent have membranes, slight cytolysis in all of these. In one hour 100 per cent had membranes. These membranes were then tested and found to be impermeable to sperm.

\*That is clotted or stuck together in clumps Cf T Brailsford Robertson, *loc cit*

*The acid-soluble substance—continued*

PROPORTION OF SUBSTANCE IN THE MIXTURE	EFFECT ON EGGS NOT PRE- VIOUSLY TREATED	EFFECT ON EGGS PREVIOUSLY TREATED WITH $\text{SrCl}_2$
1 6400	No effect after two hours	No agglutination In forty minutes 18 per cent of cloudy membranes In two hours over 80 per cent had membranes and some of these were beginning to cytolize, about 5 per cent were cytolized into "shadows"
1 12,800	No effect after two hours	In forty minutes many serrated membranes or membranes in the blister stage In these latter the membranes were hyaline, but as soon as they became complete an influx of granular material was received from the egg, rendering the membrane slightly opaque In two hours 6 per cent converted into "shadows" and over 50 per cent had membranes

*The alkali-soluble substance*

PROPORTION OF SUBSTANCE IN THE MIXTURE	EFFECT ON EGGS NOT PRE- VIOUSLY TREATED	EFFECT ON EGGS PREVIOUSLY TREATED WITH $\text{SrCl}_2$
1 400	No effect in two hours	No effect in two hours
1 800	No effect in two hours	A few eggs have small blisters in two hours, none have membranes and none are cytolized
1 1600	No effect in two hours	No effect in two hours
1 3200	No effect in two hours	No effect in two hours
1 6400	No effect in two hours	No effect in one hour In two hours 1 or 2 per cent have membranes and these eggs are in some cases cytolized
1 12,800	No effect in two hours	No effect in one hour In two hours 1 or 2 per cent have membranes and these eggs are in some cases cytolized

To 2 cc of each of these mixtures were added two drops (A) of the ripe eggs of *Strongylocentrotus purpuratus*, which had not undergone previous treatment of any kind save that of washing in sea-water or (B) of the eggs of the same female which had been previously treated by four minutes' immersion in  $\frac{3M}{8}$  SrCl<sub>2</sub>. The results obtained are tabulated on pp 7 and 8

Eggs of the same female sensitized by four minutes' immersion in  $\frac{3M}{8}$  SrCl<sub>2</sub> and then dropped into sea-water (two drops to 2 cc) were unaffected after two hours

The strontium chloride introduced with the eggs precipitated both substances and the eggs were, especially in the stronger solution, surrounded by particles of precipitate which, however, was not sufficiently dense in any case to hide the periphery of the eggs from view

We see, therefore, that the acid-soluble substance is a powerful fertilizing, agglutinating, and cytolyzing agent, whereas the alkali-soluble substance is devoid of any action which is not obviously attributable to slight contamination with the acid-soluble substance. This fact proves, if further proof were necessary, that the observed action of the acid-soluble substance is not due to contamination of the substance with the reagents employed to isolate it, for the treatment accorded to the two substances differed only in this that the one was dissolved in acid, while the other was dissolved in alkali

As I have mentioned, the "pseudo-membrane" which were formed by my first sperm-extract, differed from true fertilization-membranes not only in being permeable to sperm but in the fact that their formation did not lead to cleavage of the egg. It appeared very desirable, therefore, to ascertain whether or not the membranes formed by the action of the new sperm-extract led to cleavage of the egg. Accordingly 15 mgms of the acid-soluble substance were dissolved in 3 cc of  $\frac{N}{2}$  HCl, the solution was neutralized by the addition of 3 cc of  $\frac{N}{2}$  NaOH and rendered isotonic with sea-water by the addition of 0.9 cc of  $\frac{5M}{2}$  NaCl. This mixture was then diluted to 50 cc by the addition of filtered sea-water. The eggs of a purpuratus female were immersed for four minutes in  $\frac{3M}{8}$  SrCl<sub>2</sub> and then transferred to this mixture. The eggs were speedily and markedly agglutinated and in twenty-five minutes about 10 per cent had cloudy membranes. In forty minutes 80 per cent had membranes, and many of these were clear and hya-

line The eggs were then transferred to 50 cc of normal sea-water and left therein for ten minutes. One sample was then transferred to fresh sea-water (50 cc) and the remainder to hypertonic sea-water (50 cc sea-water + 8 cc of  $\frac{5M}{2}$  NaCl). The immediate effect of the hypertonic sea-water was to de-agglutinate the eggs. After forty, fifty, and sixty minutes, respectively, samples of the eggs were transferred from the hypertonic sea-water to normal sea-water (50 cc). They were then left in shallow vessels at room temperature overnight. After twenty-four hours it was found that, in all cases, both with and without treatment with hypertonic sea-water, about 50 per cent of the eggs had undergone division, and these were in every stage of division from the 2- to the 64-cell stage. Division had, however, proceeded very irregularly and was accompanied by cytolysis which was most marked in the eggs which had not been treated with hypertonic sea-water. A further lapse of twenty-four hours only resulted in more extreme cytolysis. The substance, therefore, is markedly poisonous for the eggs, in which respect its action strongly resembles that of saponin.<sup>7</sup>

The reasons for the failure of previous observers to extract the fertilizing agent from sperm are now clear. It is evident that it is necessary to wash the sperm free from sea-water before complete extraction is possible, and moreover, in order to obtain the most intense action it is necessary to supply not only the fertilizing but also the *motile* function of the sperm,<sup>8</sup> by carrying over the fertilizing agent into the egg through impregnation of the egg with a substance ( $SrCl_2$ ) which forms an insoluble compound with it and thus precipitates it upon the egg much as a mordant precipitates a dye upon a textile fabric.

We have seen that the fertilizing agent in sperm is precipitable by barium, strontium, and acetone, and is soluble in dilute acids. I have also found that it is thermostable, resisting an hour's exposure to 55°C. I have previously shown<sup>9</sup> that the fertilizing agent in blood-sera is precipitable by barium, strontium, and acetone and is soluble in dilute acids. Loeb has shown,<sup>10</sup> and I have con-

<sup>7</sup> Cf. J. Loeb, *Die chemische Entwicklung des tierschen Eies*, Berlin, 1909, p. 134.

<sup>8</sup> J. Loeb, *loc cit*, p. 185.

<sup>9</sup> T. Brailsford Robertson, *loc cit*.

<sup>10</sup> J. Loeb, *loc cit*, p. 187.

firmed the fact, that it is thermostable. There appears strong reason to believe, therefore, that the fertilizing agent in blood-sera is identical with the fertilizing agent in sperm.

#### CONCLUSIONS

From the spermatozoa of *Strongylocentrotus purpuratus* (carefully freed from sea-water by washing them with isotonic NaCl solution) two substances can be extracted by strongly hypotonic salt solutions containing ether which are precipitable by barium. The one is soluble in dilute acid, the other is insoluble in dilute acid but soluble in dilute alkali. Both are precipitable by acetone. The acid-soluble substance acts as a powerful fertilizing, agglutinating, and cytolyzing agent upon the eggs of *Strongylocentrotus purpuratus*. The alkali-soluble substance appears to be devoid of action.

There is strong reason for believing that the fertilizing agent in spermatozoa is identical with the fertilizing agent (oocytase) in blood-sera.





# STUDIES IN BACTERIAL METABOLISM I

By ARTHUR I KENDALL AND CHESTER J FARMER

(From the Laboratories of Biological Chemistry and Preventive Medicine and Hygiene, Harvard Medical School )

(Received for publication, April 12, 1912 )

A very fundamental and general principle of bacterial metabolism may be expressed concisely by stating that "fermentation takes precedence over putrefaction " That is to say, bacteria in general which can utilize both carbohydrate and protein, act upon the former in preference to the latter when both are present in the same medium Bacteria in common with all known living things need nitrogen to build up their bodies, it is self-evident, therefore, that even when carbohydrate is being fermented, enough protein must be broken down to satisfy their nitrogen requirements

The formulation of this principle, together with the salient facts of the evidence upon which it was based, have been discussed in detail in previous communications by one of us (A I K )<sup>1</sup> and will not be referred to here other than to state that this evidence, although well grounded and definite, is for the most part qualitative only It is the purpose of this paper to present comparative quantitative data which shall indicate the extent, and in a measure, the nature of this sparing action of carbohydrate for protein in artificial media as it is observed in certain important types of organisms of the intestinal group

Throughout this work, ammonia formation has been followed as an index for determining the rate, and to a degree, the extent of protein catabolism by these bacteria In addition to ammonia formation the rate and change of reaction of the media in terms of standard acid and alkali has also been determined, to throw additional light upon the mechanism of the decomposition of carbohydrate and protein respectively during the course of the experiments

<sup>1</sup> For literature, see Kendall *Journ of Med Res* , LV, p 117, 1911

The formol titration has also been followed throughout this work, but the results furnished no additional evidence which could be interpreted in the light of our present knowledge

**METHODS** *Preparation of media* Nutrient, sugar-free meat-juice bouillon prepared in the usual manner was used in these experiments. The preparation, adjustment of reaction and sterilization were carried out simultaneously on the entire amount of media necessary for one series of experiments. One per cent of Kahlbaum's C P dextrose was added to one-half of it, the media was then distributed in 200 cc Erlenmeyer flasks, 75 cc per flask, and the entire batch sterilized in the autoclave at the same time. The results, therefore are strictly comparable

*Organisms studied* *B. dysenteriae* (Shiga), typhosus, paratyphoid  $\beta$ , coli and proteus

*Total nitrogen* A sufficient number of total nitrogen determinations both in uninoculated and inoculated media were made to demonstrate the fact that nitrogen was neither gained nor lost during the course of the experiments

*Ammonia determination* Free ammonia was determined by a modification of the well-known Fohn air-current method <sup>2</sup>

*Determination of reaction of the media* It is commonly stated that the reaction of media in which carbohydrate is being fermented becomes progressively acid conversely, media containing only protein, or protein derivatives, becomes progressively alkaline. The amount and formation of acid and alkali respectively was recorded in terms of normal acid and alkali, using neutral red as an indicator. Neutral red was selected in preference to those indicators hitherto commonly used for this purpose, because it reacts sharply with both weak acids and weak bases at a point closely approximating absolute neutrality

*Inoculation of media* All cultures were tested for purity, rejuvenated by three successive daily transfers in sugar-free broth, and inoculated into the media with customary precautions. A series of flasks consisting of the sugar and sugar-free broths respectively, were inoculated simultaneously with each culture. These cultures were incubated at 37°C. At stated intervals one sugar

<sup>2</sup>This *Journal*, xi, p 523, 1912

and one sugar-free flask of this series were examined according to the procedures outlined above

Duplicate determinations of free ammonia were made on every culture, the greatest discrepancy amounts to 0.35 mgm of nitrogen per 100 cc of culture. Similarly, the determination of reaction is accurate within 0.25 cc normal acid or alkali per 100 cc culture.

The conditions of the experiments are purposely most favorable for putrefaction, the relatively large, free surface of the culture medium in contact with the air furnishes potentially the oxygen necessary for these organisms, both in sugar and sugar-free media. The results are consequently more significant than would have been the case if the cultures in sugar-containing media were grown anaerobically to force the bacteria to ferment carbohydrate for their oxygen supply, as is the case in the closed arm of the fermentation tube where such observations are ordinarily made.

The following tables show the results of these experiments. The figures for ammonia are expressed as milligrams of nitrogen per 100 cc of culture. The changes in reaction are likewise expressed in cubic centimeters of normal acid or alkali per 100 cc of culture in their respective media.

TABLE I

*Broth A 100 cc contains 395 mgms. total nitrogen*

	DATE	PLAIN BROTH		DEXTROSE BROTH	
		Free $\text{NH}_3$ as milligrams $\text{N}_2$ per 100 cc	Ammonia N Total N	Free $\text{NH}_3$ as milligrams $\text{N}_2$ per 100 cc	Ammonia N Total N
Control		54.60	13.83 <i>per cent</i>	53.20	13.51 <i>per cent</i>
B coli	1	59.50	15.06	54.25	13.78
	2	61.60	15.60	55.30	14.05
	3	68.60	17.37	56.00	14.17
	4	74.90	18.96	56.00	14.17
	5	72.60	18.40	57.40	14.54
	6	74.20	18.84	57.40	14.54
B proteus III	1	59.85	15.11	54.60	13.82
	2	64.05	16.23	56.00	14.17
	3	102.20	25.87	57.40	14.54
	4	107.80	27.31	58.80	14.88
	5	117.60	29.78	57.05	14.45

TABLE II

*Broth B 100 cc contains 242 mgms total nitrogen*

	DATE	PLAIN BROTH			DEXTROSE BROTH		
		Free NH <sub>3</sub> as milligrams N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc N <sub>2</sub> acid per 100 cc	Free NH <sub>3</sub> as milligrams N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc N <sub>2</sub> acid per 100 cc
Control		8 40	3 47	0 00	8 40	3 47	0 00
Proteus II	1	12 95	5 36	-0 75	10 15	4 20	+2 00
	2	15 05	6 21	-0 75	11 55	4 77	+3 25
	4	59 15	24 50	-2 25	11 55	4 77	+3 50
	6	64 05	26 50	-3 25	10 50	4 34	+3 50
	8	66 50	27 48	-3 00	10 50	4 34	+3 50
B coli	1	15 40	6 33	-0 75	7 70	3 18	+2 25
	2	16 80	6 92	-0 75	11 55	4 77	+3 25
	4	21 70	8 97	-1 25	7 70	3 18	+3 50
	6	24 50	10 12	-1 75	8 40	3 47	+3 50
	8	29 40	10 21	-3 50	9 10	3 76	+4 00
Proteus III	1	11 20	4 63	-0 50	10 15	4 20	+1 75
	2	14 00	5 79	-0 75	9 80	4 05	+3 00
	4	28 00	11 57	-1 25	9 45	3 90	+3 25
	6	46 90	19 40	-1 75	9 80	4 05	+3 25
	8	72 80	30 10	-3 50	10 50	4 20	+3 75
Paratyphoid $\beta$	1	9 80	4 05	-0 25	9 10	3 76	+2 25
	2	10 15	4 20	-0 50	8 05	3 31	+3 00
	3	11 90	4 92	-0 75	8 40	3 47	+3 00
	7	15 40	6 36	-0 50	9 80	4 05	+3 25
	9	17 50	7 24	-0 75	9 45	3 90	+3 25
	11	17 50	7 24	-1 25	9 10	3 76	+3 00
Shiga	1	8 40	3 47	+0 25	8 05	3 31	+1 75
	2	8 75	3 62	+0 25	8 75	3 62	+1 75
	3	9 10	3 76	+0 25	9 10	3 76	+2 00
	7	9 80	4 05	+0 25	9 10	3 76	+2 25
	9	11 20	4 63	$\pm$ 0 00	9 45	3 90	+2 50
	11	11 90	4 92	$\pm$ 0 00	9 80	4 05	+2 50
Typhoid	1	8 40	3 47	$\pm$ 0 00	9 80	4 05	+1 75
	2	9 10	3 76	-0 50	8 40	3 47	+2 00
	3	9 10	3 76	-0 75	9 10	3 76	+2 25
	7	11 55	4 77	-0 50	10 15	4 20	+2 25
	9	13 30	5 50	-0 75	9 80	4 05	+2 50
	11	15 40	6 36	-1 25	9 80	4 05	+2 50

A noteworthy feature is clearly brought out in this first series of experiments. Notwithstanding the fact that the ammonia production in sugar media is noticeably less than that in sugar-free media, the growths obtained in sugar media were decidedly more vigorous and extensive. The rapid increase in acid formation during the first twenty-four hours of growth is an index of the rapid fermentation of dextrose. This emphasizes the sparing action which dextrose exerts for protein in these artificial media.

#### CONCLUSIONS

These experiments show the extent, and in a measure, the nature of the sparing action which dextrose exerts for protein in ordinary media for the organisms mentioned above.

It is interesting to note that the more pathogenic bacteria exhibit less proteolytic activity measured in terms of ammonia production and alkali formation than the more saprophytic organisms.

We wish to express our indebtedness to Professor Folin for his opportune advice and criticism and to Professor Theobald Smith for cultures.



## STUDIES IN BACTERIAL METABOLISM II

By ARTHUR I KENDALL AND CHESTER J FARMER

(From the Laboratories of Biological Chemistry and Preventive Medicine and Hygiene, Harvard Medical School)

(Received for publication, April 12, 1912)

In the previous communication, the sparing action which dextrose exerts for protein in artificial media was shown quantitatively for a limited number of bacteria. It is the purpose of this paper to present additional evidence in support of this hypothesis. For the sake of clearness, the organisms discussed here will be considered separately, since the "growth curve" of each bacterium illustrates a special feature in the mechanism of this sparing action of carbohydrate for protein.

*Spirillum of Asiatic cholera* The cholera vibrio studied was obtained from a case of Asiatic cholera which developed in Boston last summer. The organism was typical in every respect, and agglutinated promptly with a specific serum of known and high potency. The unusually marked proteolytic powers of this organism distinguish it sharply from the relatively slight proteolytic activities of the pathogenic bacteria described previously. The cholera organisms are noted for their ability to breakdown protein, however, so that this observation is wholly in harmony with previous observations qualitatively, and illustrates the rapidity with which this proteolytic process proceeds. The mere trace of ammonia in the corresponding cultures containing dextrose is a striking example of the extent of the sparing action of dextrose for protein.

The cultures designated as "Pearce Farm" and "Arkansas" are, respectively, a non-virulent and a virulent culture of the hog-cholera bacillus. Professor Theobald Smith suggested that their proteolytic activities be compared. The results show clearly that the "Pearce Farm" culture (the non-virulent one) is more active proteolytically than the virulent one. This difference in proteo-





# NOTE ON THE REFRACTIVITY OF THE PRODUCTS OF THE HYDROLYSIS OF CASEIN, AND A RAPID METHOD OF DETERMINING THE RELATIVE ACTIVITY OF TRYPSIN SOLUTIONS

By T. BRAILSFORD ROBERTSON

(From the Rudolph Spreckels Physiological Laboratory of the University of California)

(Received for publication, April 13, 1912)

I have observed that the refractivity of a solution of sodium caseinate (containing  $80 \times 10^{-5}$  equivalents of sodium per gram of casein) is unaltered, within the limits of the accuracy of the determination, by digestion with trypsin. This fact suggested a rapid and simple method of determining from time to time the extent of digestion in a caseinate solution containing trypsin, namely that of precipitating the undigested casein by a known volume and concentration of acetic acid, filtering off the precipitate and measuring the refractive index of the filtrate, which contains the products of digestion.

In the experiments reported below I have employed a 2 per cent solution of sodium caseinate, neutral to phenolphthalein (= 16 cc of  $\frac{N}{16}$  NaOH diluted to 100 cc and 2 grams of casein dissolved therein), as a standard. To portions of this solution were added varying amounts of Gruebler's trypsin *puriss. sicc.* and from time to time 10 cc samples of these mixtures were taken, and the proportion of the casein which had been digested was measured by the method outlined. Preliminary experiments showed me, however, that it was very necessary to carefully adjust the concentration of the acetic acid solution employed for the precipitation to the concentration of the caseinate solution. At first I tried delivering the 10 cc samples into 10 cc each of  $\frac{N}{16}$  acetic acid, this procedure yielded cloudy opaque filtrates containing much suspended casein and totally unfit for refractometer measurements, it appeared that the acetic acid solution was too

strong On the other hand 10 cc of an acetic acid solution of the same concentration as the alkali used to dissolve the casein proved to be too weak, because, owing to the hydrolytic dissociation which sodium acetate undergoes in aqueous solution, the mixture of the 10 cc sample and the 10 cc of acetic acid solution still retained casein in solution Finally I adopted the proportion of 10 cc of the digest to 10 cc of  $\frac{N}{16}$  acetic acid which, with the precautions detailed below, yielded perfectly satisfactory results

I prepared a 2 per cent solution of casein, neutral to phenolphthalein, by stirring up casein in a dilute solution of NaOH, containing 16 cc of  $\frac{N}{16}$  NaOH per 100 cc, until the mixture was nearly clear The mixture was then filtered through a folded filter and the filtrate was divided into four portions measuring 100 cc each Each of these portions was then placed in an incubator, in a tightly stoppered Erlenmeyer flask, and allowed to stand there until it had attained the temperature of the incubator These solutions were then treated as follows

I Added 3 cc of distilled water

II Added 2 cc of distilled water + 1 cc of a 0.4 per cent freshly made up and filtered solution of Gruebler's trypsin *purissimum*

III Added 1 cc of distilled water + 2 cc of 0.4 per cent trypsin solution

IV Added 0 cc of distilled water + 3 cc of 0.4 per cent trypsin solution

To each of these mixtures were then added three drops of toluol and they were then gently shaken and replaced in the incubator From time to time, as detailed below, 10 cc samples were taken from each flask and the percentage of casein digested was estimated in the following manner

Ten cc of  $\frac{N}{16}$  acetic acid having previously been accurately measured into a small dry Erlenmeyer flask, the 10 cc sample was fairly rapidly delivered from the measuring pipette into this, the Erlenmeyer being gently rotated the while in order to uniformly mix the two solutions This mixture was immediately filtered through a small plug of glass wool, thus removing the grosser masses of casein<sup>1</sup> The opaque filtrate, containing finely

<sup>1</sup> This preliminary filtration can be omitted provided digestion has proceeded far enough

suspended casein, after standing for a few minutes to allow the casein to flocculate, is filtered through an S & S 589 "yellow ribbon" filter. Filtration is rapid and the filtrates are usually perfectly clear, or, if they are not, a second filtration will accomplish this result. The refractive index of this mixture was then determined by means of a Pulfrich refractometer reading accurately to within 1' of the angle of total reflection, a sodium flame being employed as the source of light. At the same time I determined the refractive index of a "blank" solution prepared by mixing equal volumes of 1% acetic acid and a sodium hydrate solution of the strength employed to dissolve the casein, i.e., 0.016 N. The difference between the two readings expressed the refractivity of the products of the digestion of the casein, since the refractivity of the mixed products of the tryptic digestion of casein is the same as that of the casein from which they are derived and the effect of 1 per cent casein upon the refractivity of its solution is accurately known, it is evident that this difference affords a direct measure of the amount of casein digested per 100 cc. of the digestion-mixture.

Each gram of casein dissolved in 100 cc. of an aqueous solvent increases its refractivity by 0.00152.<sup>2</sup> Consequently the difference ( $=n - n_1$ ) between the refractivity of the "blank" mixture and that of the filtrate prepared as directed above, divided by 0.00152, gives the percentage of casein which would yield, when digested, the concentration of the products of digestion which is contained in the filtrate. Since this filtrate is prepared by diluting a sample of the digest to one half with acetic acid solution, this figure must be multiplied by 2 to yield the number of grams of casein per 100 cc. of the digestion-mixture which have undergone hydrolysis.

The following were the results obtained.<sup>3</sup>

T Brailsford Robertson *Journ. of Physical Chem.*, VIII, p. 469, 1909, *Die physikalische Chemie der Proteine*, Dresden, 1912, chapter 13.

<sup>2</sup> The slight alterations in the refractivity of the "blank" solution recorded at different times are due to the fact that the temperature at which the determinations of refractivity were made varies somewhat between the one and the three-hour determinations and so forth. Since, however, the refractivity of the "blank" was determined afresh upon each occasion and at the same time as those of the mixtures under investigation, this fact did not affect the accuracy of the determinations (cf. T Brailsford Robertson, *loc. cit.*)

*After digestion for one hour at 35°*

SOLUTION	$n = \text{REFRACTIVE INDEX OF SOLUTION}^*$	$(n - n_1) = \text{DIFFERENCE BETWEEN THE REFRACTIVITY OF THE BLANK AND THAT OF THE FILTRATE}$	$2 \times \frac{n - n_1}{0.00152} = \text{GRAMS OF CASEIN DIGESTED PER 100 CC OF DIGEST}$
"Blank"	1 333558 $\pm$ 0 000078		
0 cc trypsin	1 333558 $\pm$ 0 000078	0 000000	0 0
1 cc trypsin	1 333636 $\pm$ 0 000078	0 000078 $\pm$ 0 000078	0 1 $\pm$ 0 1
2 cc trypsin	1 333714 $\pm$ 0 000078	0 000156 $\pm$ 0 000078	0 2 $\pm$ 0 1
3 cc trypsin	1 333792 $\pm$ 0 000078	0 000234 $\pm$ 0 000078	0 3 $\pm$ 0 1

\*The experimental error in the determination of the angle of total reflection by the Pulfrich refractometer is  $\pm 1$  corresponding to an error of 0 000078 in the determination of the refractive index and to an error of  $\pm 0.1$  in the grams of casein digested per 100 cc of digest

*After digestion for three hours at 35°*

"Blank"	1 333480 $\pm$ 0 000078		
0 cc trypsin	1 333480 $\pm$ 0 000078	0 000000	0 0
1 cc trypsin	1 333714 $\pm$ 0 000078	0 000234 $\pm$ 0 000078	0 3 $\pm$ 0 1
2 cc trypsin	1 333948 $\pm$ 0 000078	0 000468 $\pm$ 0 000078	0 6 $\pm$ 0 1
3 cc trypsin	1 334182 $\pm$ 0 000078	0 000702 $\pm$ 0 000078	0 9 $\pm$ 0 1

*After digestion for six hours at 35° to 36°\**

"Blank"	1 333402 $\pm$ 0 000078		
0 cc trypsin	1 333402 $\pm$ 0 000078	0 000000	0 0
1 cc trypsin	1 334026 $\pm$ 0 000078	0 000624 $\pm$ 0 000078	0 8 $\pm$ 0 1
2 cc trypsin	1 334418 $\pm$ 0 000078	0 001016 $\pm$ 0 000078	1 3 $\pm$ 0 1
3 cc trypsin	1 334734 $\pm$ 0 000078	0 001332 $\pm$ 0 000078	1 8 $\pm$ 0 1

\*The temperature of the incubator rose about 1 during this interval. The effect of this upon the rapidity of hydrolysis is very noticeable

*After digestion for twenty-three hours at 35° to 36°\**

"Blank"	1 333558 $\pm$ 0 000078		
0 cc trypsin	1 333558 $\pm$ 0 000078	0 000000	0 0
1 cc trypsin	1 334892 $\pm$ 0 000078	0 001334 $\pm$ 0 000078	1 8 $\pm$ 0 1
2 cc trypsin	1 335129 $\pm$ 0 000078	0 001571 $\pm$ 0 000078	2 1 $\pm$ 0 1
3 cc trypsin	1 335129 $\pm$ 0 000078	0 001571 $\pm$ 0 000078	2 1 $\pm$ 0 1

\*At some time during this interval the temperature fell again to 35°

After this last set of observations, it being evident that the casein in the digest containing 3 cc of trypsin solution was completely hydrolyzed,<sup>4</sup> I determined the refractivities of the mixture containing no trypsin at all, in which it is evident that no appreciable hydrolysis had taken place, and of the mixture containing 3 cc of trypsin solution and of a "blank" consisting of a solution of NaOH of the strength (0.016 N) originally employed to dissolve the casein. The following were the results obtained

solution	n = REFRACTIVE INDEX OF THE SOLUTION	(n - n <sub>1</sub> ) = DIFFERENCE BETWEEN THE REFRACTIVE INDEX OF THE DIGEST AND THAT OF THE BLANK	$\frac{n - n_1}{0.00152}$ = GRAMS OF CASEIN HYDROLYZED OR UNHYDROLYZED IN 100 CC OF DIGEST
"Blank"	1.333480 ± 0.000078		
0 cc trypsin	1.336630 ± 0.000078	0.003150 ± 0.000078	2.07 ± 0.05
3 cc trypsin	1.336630 ± 0.000078	0.003150 ± 0.000078	2.07 ± 0.05

It is evident that the refractivity of the casein is not affected by hydrolysis by trypsin.

From the results enumerated above, assuming that the hydrolysis of casein by trypsin obeys the monomolecular formula,

$\log \frac{a}{a-x} = Kt$ , where  $a$  = the initial concentration of the substrate and  $x$  the amount digested at time  $t$ ,<sup>5</sup> it is possible to estimate the values of the velocity-constant  $K$  for each of the periods of digestion and concentrations of tryptins employed. The results of this calculation are enumerated in the accompanying table, the time being expressed in hours and  $a$  (= 2.07) and  $x$  in grams per 100 cc, common logarithms being employed.

<sup>4</sup> The digests containing, respectively, 2 and 3 cc of trypsin solution yielded at this time no precipitate or opalescence on mixing with the acetic acid.

<sup>5</sup> Cf. T. Brailsford Robertson, *This Journal*, 11, p. 317, 1907; Walters *ibid.*, xi, p. 267, 1912.

TIME OF DIGESTION	TRYPSIN SOLUTION PER 100CC OF DIGEST	$\lambda$	$\lambda$ DIVIDED BY CC OF TRYPSIN SOLUTION PER 100 CC OF DIGEST
1	1	$22 \times 10^{-3}$	$22 \times 10^{-3}$
1	2	$44 \times 10^{-3}$	$22 \times 10^{-3}$
1	3	$68 \times 10^{-3}$	$23 \times 10^{-3}$
3	1	$23 \times 10^{-3}$	$23 \times 10^{-3}$
3	2	$50 \times 10^{-3}$	$25 \times 10^{-3}$
3	3	$83 \times 10^{-3}$	$28 \times 10^{-3}$
6	1	$35 \times 10^{-3}$	$35 \times 10^{-3}$
6	2	$72 \times 10^{-3}$	$36 \times 10^{-3}$
6	3	$147 \times 10^{-3}$	$49 \times 10^{-3}$
23	1	$38 \times 10^{-3}$	$38 \times 10^{-3}$

At the point indicated by the intersecting line the rise in temperature occurred

It is evident that for a given temperature the figures in the fourth column are constant, thus the rate of hydrolysis as determined by this method is directly proportional to the concentration of trypsin in the digest—a relation which has been abundantly established by more exact methods of measurement<sup>6</sup> The method is therefore adapted for the purpose for which it was designed, namely, that of rapidly determining the relative activities of trypsin solutions

While the method which I have outlined is, of course, greatly inferior in accuracy to many others, it possesses the following counterbalancing advantages (a) It is extremely rapid, (b) it is extremely simple and (c) *the experimental error, although large ( $\pm 0.1$  gram per 100 cc) is nevertheless exactly known*, it could obviously be somewhat reduced, if desired, by increasing the concentration of the caseinate solution used as substrate The method is probably not less accurate than that of Mette which has been employed with such advantage in investigations of this type, without being open to the criticism, which attaches to the method of Mette<sup>7</sup>, that phenomena of dissolution and diffusion may be confused with phenomena of hydrolysis

<sup>6</sup> Cf E H Walters *loc cit*

<sup>7</sup> A E Taylor *Univ of Calif Publ Pathol*, 1, p 7, 1904, Svante Arrhenius *Immunochemistry*, New York, 1907

## SUMMARY

1 The complete hydrolysis of sodium caseinate by trypsin does not alter the refractivity of its solution, within the limits of the accuracy of the determination

2 A method of comparing the activities of trypsin solutions, based upon this fact, is described in detail





# ON THE FORMATION OF GUANYLIC ACID FROM YEAST NUCLEIC ACID

(Preliminary Communication )

By WALTER JONES

(From the Laboratory of Physiological Chemistry, Johns Hopkins  
University )

(Received for publication, April 20, 1912 )

A demand recently arose in this laboratory for a considerable amount of guanosine, and to obtain the substance, commercial yeast nucleic acid was submitted to neutral hydrolysis in the autoclave<sup>1</sup> Although guanosine in macrocrystalline form was obtained in every trial, the yield was such that recourse was had to the more recent method adopted by Levene and LaForge<sup>2</sup> for the hydrolysis of triticonucleic acid As this second method gave results which were not different from those previously obtained, it was decided to make use of glandular ferments as hydrolytic agents Numerous experiments had already shown that with proper arrangement of the digestion, guanosine can be obtained by the action of a number of glandular extracts upon commercial yeast nucleic acid and that there is no production of the dark brown pigments which are formed in the autoclave and which render the isolation of guanosine difficult The pig's pancreas was selected as the ferment source because abundant evidence had indicated guanosine as one of the end products of the action of this tissue extract upon yeast nucleic acid

But as simple as the matter appears, a physical difficulty is encountered at the beginning which greatly impairs the usefulness of the method A warm solution of the digestion products cannot be filtered Fat globules pass through the filter in the beginning and before a clear filtrate can be obtained there is such a

<sup>1</sup> Levene and Jacobs *Berichte*, xlii, p 2704

<sup>2</sup> Levene and LaForge *ibid* , xlii, p 3166

clogging with fat that filtration ceases. On the other hand a cold solution of the digestion products can be filtered perfectly clear but guanosine, having fallen out of solution, remains on the filter. To overcome this objection it was decided to digest, cool and filter the pancreatic extract before adding the nucleic acid. Accordingly a mixture of 760 grams of trimmed and finely ground pig's pancreas with 1400 cc of distilled water was allowed to digest at 40° in a closed vessel for fourteen days with enough chloroform to prevent putrefaction. At the end of this time the product was cooled and the solution filtered. Upon standing over night there occurred a copious deposition of snow-white tyrosine needles which were filtered off. The pale yellow clear solution thus obtained was preserved for use in a closed vessel with chloroform and will be referred to as "the digested pancreatic extract".

A portion of the fluid was boiled and filtered and the free phosphoric acid present in the filtrate was determined by direct precipitation with magnesia mixture. A second portion of the boiled and filtered extract was treated with yeast nucleic acid and used as a control.

To 500 cc of the digested pancreatic extract which had been warmed to 40° were added 5 grams of yeast nucleic acid. After digestion of the material for fifteen minutes at 40°, a small portion was boiled, cooled, filtered and tested for nucleic acid by the addition of sulphuric acid, when it was found that the nucleic acid had entirely disappeared. Yet the presence of nucleic acid could be shown in the control and was apparently undiminished after two days.

After the digestion had proceeded eight hours a determination of phosphoric acid was made and compared with one made from the "digested pancreatic extract" when it was found that no phosphoric acid had been liberated from the yeast nucleic acid. Nor could the presence of free purine bases be demonstrated by the addition of silver nitrate and ammonia.

These results suggest that when an aqueous extract of pancreas is digested at 40°, the two ferments, phosphonuclease and purine nuclease,<sup>3</sup> are destroyed or greatly injured while the agent which brings about a decomposition of tetranucleotide (yeast nucleic acid) into simpler nucleotides or mononucleotides remains in

<sup>3</sup> Amberg and Jones *Zeitschr f physiol Chem*, LVIII, p 407

great activity. It has been observed that fresh serum changes the optical character of yeast nucleic acid without liberation of either phosphoric acid or purine bases,<sup>4</sup> and it is therefore possible that in fresh serum conditions exist similar to those of digested pancreatic extract.

The main portion of the product of the action of digested pancreatic extract upon yeast nucleic acid was boiled, filtered and treated at the boiling point with an excess of a solution of lead acetate, and the hot fluid was filtered from precipitated lead phosphate. Upon cooling, the pale yellow filtrate deposited a heavy granular lead compound which was filtered off, suspended in hot water and decomposed with sulphuretted hydrogen. The filtrate from lead sulphide was treated with potassium acetate and poured into an excess of alcohol. A dense flocculent white precipitate was thrown down which was washed with alcohol and dried over sulphuric acid. After purification by a repetition of the processes involved in its preparation, the substance was found to possess the following properties. An aqueous solution gives no precipitate with silver nitrate and ammonia even after the addition of an ammoniacal solution of hypoxanthine. Upon hydrolysis with dilute sulphuric acid there is formed the theoretical amount of both guanine and phosphoric acid required for the potassium salt of guanylic acid while no trace of adenine could be found among the products, at the same time a reducing substance is produced which responds to qualitative tests for pentose and the optical character of the solution becomes reversed. By a comparison of the substance with a specimen of guanylic acid prepared from the  $\beta$ -nucleoprotein of the pancreas it was found that the two substances conduct themselves throughout in precisely the same manner, so that there seems no escape from the conclusion that *the substance under discussion is the potassium salt of guanylic acid*.

The yield in each of a number of experiments was about 50 per cent of that required by theory, so that without special care it was not found difficult to prepare 25 grams of the substance in a short time—an undertaking which would require weeks or months of time if the older method of preparation be employed.

While the active agent here concerned is destroyed by heat and is more active at 40° than at 20°, it must not be supposed that we

<sup>4</sup> Amberg and Jones this *Journal*, x, p 81

are here dealing with a true catalytic agent. A given amount of digested pancreatic extract can decompose a certain definite amount of yeast nucleic acid with great rapidity but can decompose no more in any reasonable length of time. As already stated 100 cc of the extract completely decomposes a gram of yeast nucleic acid in fifteen minutes but it cannot decompose 2 grams in a week. A solution of 1.5 grams of yeast nucleic acid in 100 cc of extract was digested at 40°. After fifteen minutes a trace of nucleic acid could be shown present and after twenty-four hours the trace had not disappeared.

In the course of their polarimetric work upon the action of animal fluids upon nucleic acids Levene and Medigreceanu<sup>5</sup> were led to postulate a ferment capable of decomposing tetranucleotides into simpler nucleotides and they assign to the ferment the name "nucleinase". As the first conclusive evidence of the existence of such a ferment is presented in this paper, the writer will be permitted to suggest that the nomenclature be made conformable to that which Levene and his coworkers have adopted for the nucleic acids. The term "tetranuclease" indicates clearly a ferment which exerts its activity upon a tetranucleotide. As the active agent under consideration can exert this activity and no other (at least in so far as one of the mononucleotides is concerned) it should be termed tetranuclease.

It seems probable that there are several tetranucleases, for the active agent here considered does not exert its activity upon thymus nucleic acid. While one gram of yeast nucleic acid is completely decomposed by 100 cc of digested pancreatic extract in fifteen minutes, one-fourth the amount of thymus nucleic acid does not suffer any diminution under the same conditions in a week.

It is undoubtedly true that these two nucleic acids have the same general chemical structure; that is, they are both tetranucleotides composed of four mononucleotides which in turn are compounds of phosphoric acid with nucleosides. This close analogy was shown by Levene<sup>6</sup> and his coworkers in their chemical examination of the two nucleic acids, and the general similarity of structure is also brought out in a study of the changes which the two substances undergo in the presence of glandular extracts for the

<sup>5</sup> *This Journal*, ix, p. 395

<sup>6</sup> Levene and Mandel *Berichte*, xli, p. 1905, Levene and Jacobs *ibid*, xliii, p. 3150

characteristic conduct of thymus nucleic acid in the presence of fresh pancreatic extract<sup>7</sup> and the particular line along which the adenine group of thymus nucleic acid is changed to hypoxanthine<sup>8</sup> by the action of dog's liver are matters which can also be stated for yeast nucleic acid. Nevertheless these two nucleic acids are two different substances, and obviously because they are not composed of the same mononucleotides. They could not therefore be expected to yield identical nucleosides under any conditions. The structure of yeast nucleic acid which was assigned by Levene and Jacobs rests primarily upon the ability of these writers to isolate four nucleosides from among the products of its hydrolysis. One of these nucleosides (guanosine) possesses such physical and chemical properties that its escape from the observation of one accustomed to deal with it would seem almost impossible. But Levene and Jacobs have never obtained this substance from thymus nucleic acid. Again, Levene and Jacobs proved that the carbohydrate of yeast nucleic acid is *d*-ribose, while it has been generally conceded that the carbohydrate of thymus nucleic acid is a hexose. Finally, guanosine has been obtained in this laboratory by the action of a number of glandular extracts upon yeast nucleic acid and conditions have been found for controlling the action of both phosphonuclease and purine nuclease so that an arrangement of the digestion can be made favorable to the formation of guanosine, but no attempt to obtain the substance from thymus nucleic acid has been successful.

It is necessary to refer in this connection to an interesting but somewhat puzzling contribution of London, Schittenhelm and Wiener<sup>9</sup> who studied the decomposition of nucleic acid brought about by the intestine. They obtained from among the products a slight amount of a substance which contained organically bound phosphorus and purine base but the amount of material was too small for an exact identification. In parentheses they add "(Ammoniumsalz der Guanylsäure?)" and continue to state how they isolated and completely identified guanosine and mention a somewhat less conclusive identification of adenosine. It is interesting to note that *thymus nucleic acid* was employed in their experiments.

<sup>7</sup> Jones this *Journal*, 12, p 169

<sup>8</sup> Amberg and Jones *Zeitschr f physiol Chem*, lxxiii, p 407

<sup>9</sup> *Zeitschr f physiol Chem* lxx p 459



# THE EXCRETION OF IRON IN THE URINE IN PNEUMONIA

By EDWARD H. GOODMAN

(From the Laboratory of Dr. John H. Musser, Philadelphia, Pa.)

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*Normal excretion of iron* For many years it was a mooted question whether the urine contained iron, and among those who questioned its presence were Becquerel, Herberger, Lehmann, Schroff and Schlemmer. In 1820 Tiedemann and Gmelin pointed out that iron was constantly present, and in this they were confirmed by a host of observers. The reason for this difference of opinion lies, as suggested by Hoffmann, in the too small amounts of urine examined by Becquerel and his followers.<sup>1</sup> In the early estimations of iron, widely varying figures were obtained, but since the report of Neumann<sup>2</sup> and of Neumann and Mayer<sup>3</sup> it is generally accepted that the normal twenty-four-hour output of iron is about 1 milligram.

*Excretion of iron under pathological conditions* The excretion of iron in disease has been more or less carefully studied, but one must view with some scepticism results reported before the description by Neumann of his method. Diseases of the blood have, as might be expected, received the greatest attention although evidences of sporadic interest in other diseases are not wanting.

*Chlorosis* Zander<sup>4</sup> found a decided decrease in the urinary iron although he gives no definite figures. Quincke<sup>5</sup> showed that the amount of iron excreted varied with the stage of the disease, in

<sup>1</sup> For literature see Kennerknecht *Virchow's Archiv*, ccv, p. 89, 1911.

<sup>2</sup> Neumann *Zeitschr. f. physiol. Chem.*, xxxvii, p. 114, 1903.

<sup>3</sup> Neumann and Mayer *Ibid.*, xxxvii, p. 143, 1903.

<sup>4</sup> Cited from Kennerknecht *loc. cit.*

<sup>5</sup> Cited from Kennerknecht *loc. cit.*



the beginning, there was an increase over normal, while in the later stages there was a diminution, and as Kennerknecht says, this may explain why Lehmann<sup>6</sup> found an increase Hunter<sup>7</sup> reported a marked reduction in iron, and ten years later Jolles and Winkler<sup>8</sup> confirmed this finding only in less degree

*Pernicious anemia* In 1890 Hunter studied the iron output in a case of pernicious anemia and found that although there was an increased output in the urine the maximum was reached three weeks before death when the anemia was most intense At this time 32.26 mgms were found, and a week before exitus, this had dropped to 6.52 mgms while two days before death there was only 1 mgm Damaskin<sup>9</sup> reported increased amounts of iron, up to 3.08 mgms a day, and Hopkins<sup>10</sup> found variable amounts, the highest being 8.3 mgms on one day, while shortly afterwards only traces were found

Kennerknecht<sup>11</sup> found that the amounts of iron varied according to the stage of the disease analogous to the finding of Quincke in chlorosis, sometimes being increased and sometimes being decreased

*Leukemia* The study of the iron in the urine in this disease has received some attention Jolles and Winkler reported a decided increase in the excretion of iron and Hoffmann a moderate increase Mayer found 3.407 mgms using Neumann's method Kennerknecht studied the effect of X-rays on the excretion of iron during the treatment of myelogenous leukemia by this method, and found that the already increased amount of urinary iron was increased still further by the use of the Roentgen ray

*Nephritis* Damaskin found a variance in iron depending on the form of nephritis present, it being slightly increased or normal in interstitial nephritis and greatly increased in the parenchymatous form Jolles and Winkler found it increased in every form of nephritis Corroboration of the increased amount of iron in chronic interstitial nephritis is offered by Neumann and Mayer

<sup>6</sup> Cited from Kennerknecht *loc cit*

<sup>7</sup> Hunter *Brit Med Journ*, July 5, p 1, 1890

<sup>8</sup> Jolles und Winkler *Arch f exp Path u Pharm*, xlv, p 464, 1900

<sup>9</sup> Cited from Kennerknecht *loc cit*

<sup>10</sup> Hopkins *Guy's Hospital Reports*, 1, p 373, 1894

<sup>11</sup> *Loc cit*

*Miscellaneous diseases*

Anemia	Decrease	}	Damaskin
Pneumonia	Decrease		
Malaria	Increase	}	Colasanti and Jacoangeli Damaskin
Obstructive jaundice	Normal		
Catarrhal jaundice	Slight decrease	}	Jolles and Winkler
Alimentary glycosuria	Normal		
Gout	Increase		
Carcinoma of stomach	Increase		
Fever	Decrease		
Fever	Increase	}	Neumann and Mayer Colasanti and Jacoangeli
Fever	Increase		
Typhoid fever	Increase	}	Neumann and Mayer
Erysipelas	Increase		
Cholelithiasis	Increase		
Cirrhosis of the liver	Increase		
Alcoholism-chronic	Increase		

In all these articles referred to above there is but one mention of the study of the urinary iron in pneumonia. This is striking inasmuch as one would expect to find very interesting results during the course of the infection.

Our interest in morbid process, as far as the latter concerns us in this paper, is especially concentrated on the erythrocytes. In health, as is well known, the red blood cells after a certain period of life, are eventually destroyed, and following their dissolution are probably taken up by the leucocytes and by them transported to the liver, spleen and bone marrow. Of the iron thus liberated, a part finds its way into new erythrocytes and a part is excreted, and for this excretion two possibilities are recognized by von Noorden,<sup>12</sup> the "mechanische Entfuhrung" and "besondere Zerfallsvorgange." In health the latter plays no rôle, and the quantity of iron which is eliminated in twenty-four hours is derived for the most part from the iron contained in the food and in lesser degree from the destruction of worn-out erythrocytes.

If we revert to the pathology of a pneumococcus infection of the lung it will be seen that during the first stage of the disease—the stage of congestion—the erythrocytes may be held to be hyperfunctioning, and having more work to do, their period of activity is curtailed, their life cycle is shorter, and they are destroyed earlier

<sup>12</sup> Cf. Neumann *loc cit*

than is a red cell existing under normal conditions. In the stage of hepatization owing to the segregation of gargantuan numbers of erythrocytes, there should be a withdrawal of cells from the circulation and hence an oligocythemia, which has indeed been described by Bollinger (Osler<sup>13</sup>). When autolysis of the exudate sets in (stage of resolution), the iron pigment of the erythrocytes is set free, and there should be, hypothetically, what might be termed hypersideremia. In this rapid holocaust of red cells, we have von Noorden's "besondere Zerfallsvorgänge" typically exemplified. The iron metabolism, theoretically be it remembered, should therefore be such that in the early stage one would find perhaps a slight increase in the urinary iron, in the middle stages, a decrease, with the crisis and thereafter, a marked increase. Evidence of the last named is seen in the icterus which often accompanies pneumonia.

The urine was collected for twenty-four hours and the iron estimated by the method of Neumann<sup>14</sup> using 500 cc of urine when the daily amount of urine permitted.

We have studied four cases occurring in the University Hospital. Some we saw early in the disease and some later, but it was unfortunate that we were not able to continue the investigations over a longer period of time following the crisis. As far as possible, only cases which promised to have a favorable outcome were chosen, and only those whose pulmonary signs gave no room for doubt as to the correct diagnosis of croupous pneumonia. Unfortunately, examination of the stools could not be undertaken.

We are indebted to Dr. Stengel for the privilege of studying his cases, the other cases occurring on the service of my lamented Chief, Dr. John H. Musser.

During the examination the patient received no iron medication and was on a diet, consisting for the most part of milk, which was practically iron-free.

<sup>13</sup> Osler *Practice of Medicine* (sixth edition), p. 177.

<sup>14</sup> Cf. Kennerknecht *loc. cit.*

*Case 1—Eduard F Service of Dr Musser, University Hospital*

DATE	DAY OF ILLNESS	URINE	IRON
1912		cc	mg
January 3-4	5	970	4 040
January 4-5	6	1000	2 20
January 5-6	7	510	1 24
January 6-7	8	1380	1 299
January 7-8	9	1160	0 635
January 8-9*	10 (crisis)	800	4 676
January 9-10	First day after crisis	920	3 650
January 10-11	Second day after crisis	1170	1 562

On this day patient looked as if he were going to have his crisis but he developed erysipelas of his nose which influenced the temperature curve and the estimations were discontinued

*Case 2—Lillian J Service of Dr Stengel, University Hospital*

January 30-31	8	1150	1 4
January 31-February 1	9	220	1 2
February 1-2	10	560	0 38
February 2-3	11 (crisis)	540	0 638
February 3-4	First day after crisis	295	0 10
February 4-5	Second day after crisis	510	0 800

*Case 3—Thomas F Service of Dr Musser, University Hospital*

February 4-5	4	2480	0 25
February 5-6	5 (crisis)	2200	1 26
February 6-7	First day after crisis	980	2 00

*Case 4—Edward S Service of Dr Stengel, University Hospital*

February 28-29	5	770	0 6
February 29			
March 1	6	920	1 0
March 1-2	7 (crisis)	630	2 38
March 2-3	First day after crisis	600	1 60

A study of these figures makes apparent the distinct curve of urinary iron excretion during the course of pneumonia, there being hyposiduria during the height of the infection, and in every instance a relative hypersiduria (in most cases an absolute hypersiduria) at the crisis or during the first day following. It would be of great interest to study the iron metabolism in a case of pneumonia with icterus as this might be able to shed some light on the question of hemolytic jaundice. Unfortunately no such case presented itself while the study of these cases was in progress.

## STUDIES IN THE ACTION OF TRYPSIN

### II (a) ON THE INFLUENCE OF THE PRODUCTS OF HYDROLYSIS UPON THE RATE OF HYDROLYSIS OF CASEIN BY TRYPSIN, (b) THE AUTOHYDROLYSIS OF THE CASEINATES

B. E. H. WALTERS

(From the Rudolph Spreckels Physiological Laboratory of the University  
of California)

(Received for publication, May 2, 1912)

In a previous communication<sup>1</sup> it was shown that the basic caseinates of the alkalis and alkaline earths are hydrolyzed by trypsin with approximately equal velocities for all concentrations of substrate between 0.2 and 4 per cent and that the rate of hydrolysis obeys the molecular reaction formula,  $\log_{10} \frac{a}{a-x} = Kt$ , during

all stages of the reaction and also that the influence of the trypsin in accelerating the reaction is that of multiplying the velocity constant  $K$  in the equation by a factor proportional to the concentration of the enzyme. It was inferred that the products of hydrolysis had little or no influence upon the rate of hydrolysis.

The depressant action of the products of hydrolysis has been shown by Bayliss,<sup>2</sup> and Hedén.<sup>3</sup> Bayliss used 5 per cent solutions of casein in each digest to which were added equal amounts of trypsin and varying amounts of concentrated digestion products. These substances were found to have a retarding action which increased with increasing amounts of the products. He considered this effect due in part to a combination occurring between the

<sup>1</sup> E. H. Walters, *This Journal*, vi, p. 267, 1912.

<sup>2</sup> Bayliss, *Arch. sci. biol.* (St. Petersburg, 1904), 11 supplement, p. 261, reprinted in the *Collected papers of the Physiological Laboratory, University College, London*, xiii, 1903-5.

<sup>3</sup> Hedén, *Journ. of Physiol.*, xxxii, p. 468, 1904, *Ibid.*, xxxiv, p. 370, 1906.

trypsin and products, thus actually reducing the active mass of the ferment

The experiment which I am about to describe shows that this impeding action has been overstated and that the rapid falling off in the velocity constant in Bayliss' experiments was undoubtedly due to the very rapid destruction of the trypsin by the high alkalinity of his solutions. He used 2 cc of  $N$  ammonia to 6 cc of the system. In the paper referred to above a number of experiments upon the hydrolysis of casein by trypsin were reported which indicate that the influence of the products of hydrolysis upon the rate of hydrolysis is only very slight. It appeared important, however, to determine accurately the extent of this influence and it was with this object that the following experiment was undertaken.

The casein and trypsin employed were the same as were used in my former experiments and the experimental procedure was precisely identical. The actual experiment was carried out in the following manner.

A 0.5 per cent solution of basic sodium caseinate was made by dissolving 36 grams of purified casein in 288 cc of  $\frac{N}{10}$  NaOH and diluting to 7200 cc with distilled water, as free from carbon dioxide and ammonia as could be accomplished by boiling, and 100 cc placed in Erlenmeyer flasks of 200 cc capacity provided with tightly fitting rubber stoppers. These solutions are neutral to phenolphthalein, i.e., faintly alkaline, the  $OH^-$  concentration being  $10^{-5} N$ . Taylor<sup>4</sup> and Robertson and Schmidt<sup>5</sup> have found that trypsin acts most energetically in solutions of this alkalinity.

A strong solution of the digestion products was prepared by allowing 1 liter of a 2 per cent sterile solution of basic sodium caseinate to digest for a month. This solution was then heated to boiling to destroy the last traces of trypsin that might possibly be present and evaporated down to 400 cc on an air bath heated by an electric bulb at 55°C. This concentrated solution of the products of the tryptic digestion of casein was a clear yellow-brown syrupy liquid which is just about saturated with respect to tyrosine.

To each of a set of six flasks of the casein solution were added 0.2 cc of toluol, 1 cc of a 1 per cent solution of the trypsin which had been filtered, and 1 cc of the solution of the products. To another set of six flasks were added the same quantities of toluol and trypsin and 2 cc of the pro-

<sup>4</sup> Taylor *Univ of Calif Pub Pathol*, 1, p 251, 1907

<sup>5</sup> T. Brailsford Robertson and C. L. A. Schmidt *This Journal*, v, p 31,

ducts and so on up to 10 cc. A control experiment of six flasks containing none of the products was made simultaneously. In addition to this, 25 cc of products were added to each of the two digests containing the same amount of trypsin as the others and allowed to incubate for three hours when the undigested casein was measured. In order to determine the effect of diluting the system, 10 cc of pure water were added to two digests and 25 cc to two others, the same quantity of trypsin was added to each and the whole allowed to digest for three hours. We have then eleven sets of six digests, each containing varying amounts of the products of hydrolysis from 0 to 10 cc, one set of two flasks, each containing 25 cc, and two sets of two flasks, each containing none of the products, but instead, 10 and 25 cc of pure water respectively. The experiments were carried out at the same temperature simultaneously so that the concentration and activity of the trypsin would be the same in each.

The solutions were warmed separately to the temperature of the incubator and the products and the trypsin added to the casein digests and allowed to incubate at  $37.5^{\circ}\text{C} \pm 0.5^{\circ}$ . Two flasks from each set were taken out after one, two, and three hours and the casein determined in each by precipitating with acetic acid and subsequently estimating the quantity of nitrogen in the precipitate.\*

The initial amount of casein in each digest was found from the mean of two determinations to be 428.5 mgms. The results are tabulated in the following tables.

TABLE I

<i>Set A—0 cc products</i>			
HOURS	$a-x$	$\log_{10} \frac{a}{a-x}$	$K$
0	428.5		
1	243.0	0.24634	$25 \times 10^{-2}$
2	126.5	0.52986	$26.5 \times 10^{-2}$
3	71.1	0.78008	$26 \times 10^{-2}$
<i>Set B—1 cc products</i>			
0	428.5		
1	252.5	0.22969	$23 \times 10^{-2}$
2	140.9	0.48304	$24 \times 10^{-2}$
3	73.8	0.76389	$25.5 \times 10^{-2}$
<i>Set C—2 cc products</i>			
0	428.5		
1	258.3	0.21983	$22 \times 10^{-2}$
2	148.1	0.46139	$23 \times 10^{-2}$
3	78.8	0.73542	$24.5 \times 10^{-2}$

\* The details of the method are given in the first paper of these studies to which reference has been made elsewhere.



## Action of Trypsin

TABLE I—Continued

*Set D—3 cc products*

HOURS	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$
0	428 5		
1	253 8	0 22746	$23 \times 10^{-2}$
2	147 6	0 46286	$23 \times 10^{-2}$
3	78 8	0 73542	$24.5 \times 10^{-2}$

*Set E—4 cc products*

0	428 5		
1	257 4	0 22134	$22 \times 10^{-2}$
2	149 4	0 45760	$23 \times 10^{-2}$
3	99 9	0 63238	$21 \times 10^{-2}$

*Set F—5cc products*

0	428 5		
1	278 1	0 18775	$19 \times 10^{-2}$
2	179 6	0 37764	$19 \times 10^{-2}$
3	107 1	0 60216	$20 \times 10^{-2}$

*Set G—6 cc products*

0	428 5		
1	277 2	0 18916	$19 \times 10^{-2}$
2	176 0	0 38644	$19 \times 10^{-2}$
3	113 0	0 57887	$19 \times 10^{-2}$

*Set H—7 cc products*

0	428 5		
1	272 3	0 19690	$19.5 \times 10^{-2}$
2	188 1	0 35756	$18 \times 10^{-2}$
3	120 6	0 55060	$18 \times 10^{-2}$

*Set I—8 cc products*

0	428 5		
1	278 0	0 18791	$19 \times 10^{-2}$
2	177 8	0 38202	$19 \times 10^{-2}$
3	122 9	0 54240	$18 \times 10^{-2}$

TABLE I—Continued  
*Set J—9 cc products*

HOURS	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$k$
0	428 5		
1	277 2	0 18916	$19 \times 10^{-2}$
2	200 7	0 32940	$16.5 \times 10^{-2}$
3	133 2	0 50745	$17 \times 10^{-2}$

*Set K—10 cc products*

0	428 5		
1	272 7	0 19626	$19.5 \times 10^{-2}$
2	194 0	0 34415	$17 \times 10^{-2}$
3	144 0	0 47359	$16 \times 10^{-2}$

*Set L—25 cc products*

0	428 5		
3	200 0	0 33092	$11 \times 10^{-2}$

*Set M—10 cc H<sub>2</sub>O*

0	428 5		
3	77 5	0 74265	$24.5 \times 10^{-2}$

*Set N—25 cc H<sub>2</sub>O*

0	428 5		
3	79 0	0 73432	$24.5 \times 10^{-2}$

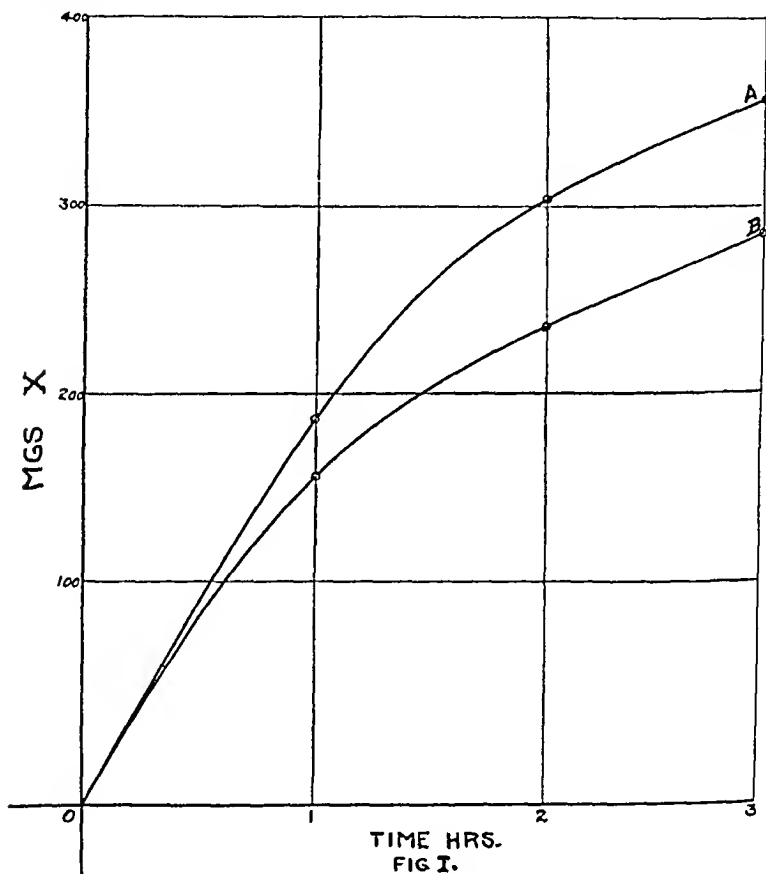
The above results show that the products of hydrolysis have only a very slight impeding action on the rate of hydrolysis. This fact is shown more clearly in fig I in which  $A$  represents the normal change and  $B$  the course of the reaction under the influence of the most concentrated products.

#### THE AUTOHYDROLYSIS OF THE CASEINATES

In the researches on casein little importance has been assigned to the fact that it undergoes fairly rapid autohydrolysis in neutral sterile solution. Robertson<sup>7</sup> made a few experiments in this con-

<sup>7</sup> T. Brailsford Robertson, *Univ of Calif Pub Physiol*, iii, p 174, 1909 (see footnote).

nection and found for a 2.8 per cent solution of neutral sodium caseinate that the velocity constant (calculated from the monomolecular reaction formula, common logarithms being employed and the time expressed in hours) was, after twenty days at 36°C, 0.000518



It appeared important to follow up this observation and to determine the relation between the time of the autohydrolysis and the amount of protein transformed, and also to determine the relation between the nature of the base combined with the casein and the rate of autohydrolysis. Unfortunately experiments of this character occupy a considerably long period of time and the

results obtained are not as satisfying as would be desired, due to certain uncontrollable errors that do not enter into short experiments. However the results of the experiments carried out are very suggestive and serve to show the magnitudes and general course of the reactions.

EXPERIMENT 1 Solutions of the neutral caseinates of lithium, sodium and potassium were made in the usual way such that the proportion of base to casein was  $50 \times 10^{-5}$  equivalents per gram. One hundred cubic centimeters of the respective solutions were placed in Erlenmeyer flasks of 200 cc. capacity, a few drops of toluol were added and the flasks tightly closed with clean rubber stoppers and allowed to digest at  $37.5^\circ\text{C} \pm 1^\circ$ . The following results were obtained.

TABLE II  
*Autohydrolysis of neutral caseinates ( $37.5^\circ \pm 1^\circ$ )*  
*Neutral lithium caseinate*

TIME IN HOURS	$x$	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$	$K \times 10^3$
0		188.0			
96	11.8	176.2	0.02815	0.0002932	29.5
192	17.9	170.1	0.04346	0.0002263	22.5

*Neutral sodium caseinate*

0		185.4			
96	12.6	172.8	0.03057	0.0003184	32
192	21.4	164.0	0.05327	0.0002774	28

*Neutral potassium caseinate*

0		188.1			
96	11.7	176.4	0.02789	0.0002905	29
192	18.9	169.2	0.04599	0.0002395	24

The figures in the column headed  $a - x$  are each the mean of three determinations. These solutions are neutral to litmus and therefore approach as nearly as possible absolute neutrality. It is improbable, therefore, that the comparatively rapid autohydrolysis is due entirely to H and OH ions. The above results show that the neutral caseinates of Li, Na, and K are hydrolyzed with approximately equal velocities. It will also be observed that they do not obey strictly the monomolecular reaction for-

mula as the values of the constants diminish as the reaction proceeds

EXPERIMENT 2 Solutions of the basic caseinates of Li and Na were prepared in the usual way such that the proportion of the base to casein was  $80 \times 10^{-5}$  equivalents per gram. These solutions are neutral to phenolphthalein and have an  $\text{OH}^-$  concentration of  $10^{-5} \text{ N}$ . The experiment was carried out in precisely the same manner as the previous one. The following are the results obtained. The figures in the column headed  $a-x$  are each the mean of three determinations.

TABLE III  
*Basic lithium caseinate*  $37.5^\circ\text{C} \pm 1^\circ$

TIME IN HOURS	$x$	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	177			
45	7	170	0.01752	0.0003893	39
137	15	162	0.03845	0.0002807	28
281	23	154	0.06045	0.0002151	21.5

*Basic sodium caseinate*  $37.5^\circ\text{C} \pm 1^\circ$

TIME IN HOURS	$x$	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	175			
45	6	169	0.01517	0.0003371	34
137	14	161	0.04621	0.0003373	34
281	23	152	0.06120	0.0002178	22

EXPERIMENT 3 This experiment was carried out in exactly the same way with the basic caseinates of Ca and Ba.

TABLE IV  
*Basic calcium caseinate*  $38.5^\circ\text{C} \pm 1^\circ$

TIME IN HOURS	$x$	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	153			
144	40	113	0.13162	0.0009139	91.5
288	48	105	0.16350	0.0005677	57.0
432	60	93	0.21621	0.0005005	50.0

*Basic barium caseinate*

TIME IN HOURS	$x$	$a-x$	$\text{Log}_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	153			
144	40	113	0.13151	0.0009139	91.5
288	51	102	0.17609	0.0006115	61.0
432	66	87	0.24517	0.0005675	56.5

The results tabulated in the above tables show that the basic caseinates of Li and Na are hydrolyzed with equal velocities and that the velocity constant is slightly higher than that obtained for the neutral caseinates. The rate of autohydrolysis of the basic caseinates of Ca and Ba is about three times as great as that of Li and Na. This fact indicates that some factors other than H or OH ions accelerate the reaction. The above experiments on the autohydrolysis were all carried out with the same preparation of casein so that the results are in every way comparable. It will be observed, also, that in all cases the values of the velocity constants diminish as the reaction proceeds, thus indicating that the reaction is a reversible one and that the point of equilibrium is attained before complete hydrolysis. It appeared, therefore, that probably the accumulation of the products of the reaction caused the falling off in the velocity constant. I therefore set out to determine this influence in the following way.

EXPERIMENT 4. A 0.4 per cent solution of basic sodium caseinate was prepared and 100 cc digests prepared in the usual way except that 150 cc reagent bottles were used instead of Erlenmeyer flasks. A strong solution of the products of the tryptic digestion of casein was prepared as described in the first part of this paper such that 10 cc contained the products that would be obtained from the complete hydrolysis of each digest. Various amounts of this solution were added to the solutions under investigation and the digestion carried out at  $73^{\circ}\text{C} \pm 1^{\circ}$ . Two experiments of the same general character were carried out at different times. The results obtained are tabulated in the following tables.

TABLE V  
0 cc products  $73^{\circ}\text{C} \pm 1^{\circ}$

TIME IN HOURS	$x$	$a-x$	$\log_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	360			
24	41	319	0.05251	0.002188	22
48	63	297	0.08354	0.001740	17.5
72	89	271	0.12333	0.001361	13.5

7 cc products

TIME IN HOURS	$x$	$a-x$	$\log_{10} \frac{a}{a-x}$	$K$	$K \times 10^5$
0	0	360			
24	36	324	0.04575	0.001906	19
48	62	298	0.08208	0.001710	17

forty-eight hours standing The filtrate from the mixture, however, contains the active substance which is capable of hydrolyzing casein If the solution is heated to a higher temperature (boiling) the precipitate separates out immediately

3 Neutral caseinates of Li, Na and K in sterile solution undergo comparatively rapid autohydrolysis, approximately 5 per cent of the substrate being hydrolyzed in ninety-six hours at 37.5°C The basic caseinates of the same bases undergo autohydrolysis at a slightly higher velocity

4 The velocity constant for the autohydrolysis of the basic caseinates of Ca and Ba is about three times as great as that for Li and Na thus indicating that some factor other than the H or OH ions plays a part in accelerating the autohydrolysis Upon long standing it was observed that these salts had a very slight tendency to coagulate at a temperature of 36°C when strong solutions were employed In comparatively weak solutions this change was not observed

5 The velocity constant for the autohydrolysis of the neutral and basic caseinates of the alkalis and alkaline earths calculated from the monomolecular formula,  $\text{Log}_{10} \frac{a}{a-x} = Kt$ , diminishes as the reaction proceeds

6 The influence of the products of the tryptic digestion of casein upon the rate of autohydrolysis of basic sodium caseinate at 73°C  $\pm 1^\circ$  is only very slight and does not account for the rapid falling off in the velocity constant in the normal reaction of autohydrolysis If the phenomenon is due to the influence of the products of the reaction then the products of the tryptic digestion of casein are not identical with those of the autohydrolysis

7 The temperature coefficient for the autohydrolysis of basic sodium caseinate between 37° and 73°C is approximately 7

8 The failure to get complete hydrolysis of the neutral and basic caseinates in the absence of a ferment or any other catalyser indicates that in the process of the hydrolysis of casein by trypsin, which is a monomolecular reaction, the station of equilibrium is shifted in the direction protein  $\rightarrow$  products by the enzyme

It is a pleasure to thank Dr T Brailsford Robertson for his very valuable advice and unremitting interest during the progress of these studies

# ON THE PRESENCE OF ACTIVE PRINCIPLES IN THE THYROID AND SUPRARENAL GLANDS BEFORE AND AFTER BIRTH

(SECOND PAPER)

By FREDERIC FENGER

(From the Chemical Research Laboratory of Armour and Company, Chicago)

(Received for publication, May 6, 1912)

In the first paper on this subject it was shown that both the thyroid and suprarenal glands from beef, hog and sheep contain their active principles not only at time of birth of the animal but also in the fetus. In the first series of experiments no distinction was made as to the sex of the fetus nor was the relation between the glands of the pregnant animals and those of the corresponding fetuses considered. Another important factor which has not been taken into consideration is the relative activity of the ductless glands of the fetus and of the corresponding digestive glands. As far as the available material permitted, these problems are discussed in the present paper.

With reference to the age and sex of the fetus two important questions naturally suggest themselves, namely, how soon after conception do the glands of the fetus contain active principles, and does the content of active principles in these glands bear any relation to the sex of the fetus? In trying to answer these questions the following set of experiments was made. The work was limited to glands from cattle, as the fetuses of sheep and hogs were too small for practical purposes.

Three stages of age were selected, namely, the fetus six to twelve weeks old, the fetus four to five months old, and suckling calves six weeks old. The glands from the different sexes of the animals were prepared for analysis by trimming and weighing, mincing, drying at 35° to 50°C to constant weight, and freeing from fat



by extracting with petroleum ether in the Soxhlet's extractor - All the determinations were made in duplicate on composite samples of glands from the number of fetuses and young animals specified in the tabulation below. The thyroid as well as the suprarenal glands were obtained from the same animal in all instances.

TABLE 1

	FETUS SIX TO TWELVE WEEKS OLD		FETUS FOUR TO FIVE MONTHS OLD		SUCKLING CALVES SIX WEEKS OLD	
	Male	Female	Male	Female	Male	Female
Number of fetuses	20	20	6	9	17	23
Average weight of fetuses, grams	623	626	5320	5348		
Average weight of thyroid glands, grams	0 22	0 29	1 90	2 60	8 20	8 00
Average weight of suprarenal glands, grams	0 07	0 08	0 36	0 40	1 70	1 80
Iodine in desic- cated fat-free thyroids, per cent	0 07	0 08	0 31	0 20	0 21	0 25
Epinephrin in desiccated fat- free supra- renals, per cent	3 20	3 40	3 80	4 20	2 60	3 00

The above figures show definitely that both the thyroid and the suprarenals of the fetus contain active principles within a few weeks after conception. Also, the epinephrin in the suprarenals seems to be present in larger and more uniform proportions than the iodine in the thyroids. The amount of iodine in the thyroids does not seem to have any definite relation to the difference in sex, whereas the quantities of epinephrin in the suprarenals seem to be slightly higher throughout in the female than in the male gland. It will, however, require more extended investigations to decide whether these observations will hold good throughout, or are purely accidental.

In order to ascertain if the thyroids from sheep and hogs are normally enlarged and the secretions increased during pregnancy, and whether any definite relationship exists between the amount of iodine in the glands of the pregnant animals and those of the corresponding fetuses, the following series of experiments was conducted on glands from pregnant hogs and sheep and the corresponding fetuses. Four sets of analyses were made at intervals of a week, two on sheep glands and two on hog glands. This precaution was taken to insure variation in sources of the stock and to eliminate possible influences of local conditions upon the animals coming from the same locality. The glands were prepared for analysis as described before.

TABLE 2  
*Sheep thyroids*

	FETUS TWO TO FOUR MONTHS OLD		FETUS THREE TO FOUR AND A HALF MONTHS OLD	
	Ewe	Fetus	Ewe	Fetus
Number of glands	10	17	8	8
Maximum weight per gland, grams	16.8	1.3	8.7	2.3
Minimum weight per gland, grams	3.4	0.4	1.8	1.1
Average weight per gland, grams	6.6	0.8	4.7	1.7
Moisture, per cent	71.5	80.0	60.8	82.7
Soluble in petroleum ether, per cent	1.6	0.7	6.0	0.8
Desiccated fat-free gland, per cent	26.9	19.3	33.2	16.5
Iodine in desiccated fat-free gland, per cent	0.53	0.36	0.28	0.09

TABLE 3  
*Hog thyroids*

	FETUS, THREE MONTHS OLD		FETUS THREE MONTHS OLD	
	Sow	Fetus	Sow	Fetus
Number of glands	1	10	4	30
Maximum weight per gland, grams	11.1	0.34	13.0	0.30
Minimum weight per gland, grams	11.1	0.27	6.0	0.15
Average weight per gland, grams	11.1	0.30	9.5	0.21
Moisture, per cent	64.0	85.7	67.4	81.3
Soluble in petroleum ether, per cent	15.2	1.0	8.7	1.5
Desiccated fat-free gland, per cent	20.8	13.3	23.9	17.2
Iodine in desiccated fat-free gland, per cent	0.24	0.11	0.45	0.32

The results obtained above show decided individual variations in the amount of iodine present in the thyroid gland of the hog and sheep. They also indicate a definite tendency in the relation between the amount of iodine in the glands of the pregnant animals and those of the corresponding fetuses. The thyroid gland during pregnancy of the hog and sheep does not show any material increase in weight or size. The moisture content, however, is somewhat lower, and the amount of iodine, in at least some instances, higher than in the normal glands of full-grown, non-pregnant hogs and sheep.

So far the writer has dealt with the ductless glands exclusively, and while it has been proven satisfactorily that in the fetus, at least, the thyroid and suprarenals, contain their active principles long before the time of birth, the possibility still suggested itself that a similar condition might exist in the digestive glands.

The digestive glands are not active in the fetus, at least they do not exercise the same function before birth as they do after, and if we, therefore, should find the same active principles in them before birth, we might reason by analogy that the presence of active principle in a gland does not necessarily mean activity on the part of the gland. If this should be the case it would conflict with the idea expressed by the writer that the amounts of active principle present in the thyroid and suprarenals are indications of the relative activity and that since the secretions of these glands are necessary, not merely for the maintenance of life and healthy metabolism, but also to govern the growth of the young animal, we might reasonably expect to find these glands active, not merely at the time of birth, but also in the fetus, especially as these glands only produce internal secretions, which as far as we know, do not enter the alimentary tract.<sup>1</sup>

Of the digestive glands the hog pancreas was found most suitable for investigation, partly on account of the size, and also because the methods for the assay of the diastatic ferment are, in practice, best known as applied to the product obtained from the hog. The pancreas from full-grown hogs as well as from the fetuses were collected simultaneously with the thyroid glands.

<sup>1</sup>This *Journal*, xi, p. 489, 1912

The U S Pharmacopocia (1905) test for diastatic power of pancreatin was applied both to the desiccated fat-free pancreas of the hogs and the fetuses. It was found that while the pancreas from the full-grown animals showed a diastatic power of 1.40 in five minutes, the pancreas from the fetuses showed a diastatic power of only 1.25 in one hundred and twenty minutes, or a mere trace. Desiccated pancreas testing 1.40 in five minutes in diastatic power will test approximately 1.350 in one hundred and twenty minutes. In this instance, therefore, the pancreas from the full-grown hogs showed a diastatic power approximately one hundred and forty times higher than the pancreas from the fetuses, while the thyroids from pregnant hogs contained only from one-half to one-third more iodine than the corresponding fetuses.

When these facts are taken into consideration, and when it is borne in mind that hepatic and renal activities are established at an early date during intra-uterine life, it seems reasonable by analogy to assume that both the thyroid and suprarenals of the fetus take a distinct and active part in the growth and development of the unborn animal.



# ON THE NATURE OF THE SO-CALLED ARTIFICIAL GLOBULIN

By ROBERT BANKS GIBSON

(From the Department of Physiology and Pharmacology, University of Minnesota)

(Received for publication, May 6, 1912)

In a paper published several years ago, Moll<sup>1</sup> reported the apparent conversion of serum albumin into serum globulin. This change was accomplished by heating the albumin at 55° to 60°C for an hour after the addition of a little dilute alkali. In the process, a part of the sulphur of the protein was split off, and the resulting products had the properties and the elementary composition of the so-called "eu-" and "pseudo-" globulins.

Moll considered that his artificial globulins were identical with the serum globulins. He further suggested that the well known increase of the serum globulin content of the blood,<sup>2</sup> along with coincident fall in the serum albumin, might be explained as the result of a similar change.

The results obtained by Moll were at first generally accepted. Hammarsten<sup>3</sup> has pointed out, however, that a true conversion of the serum albumin into serum globulin is *a priori* out of the question, for by the action of a weak alkali on the albumin, which yields no glycocoll on cleavage, we cannot expect to obtain a protein which contains glycocoll.

Recent observations on the formation of hippuric acid, indicate that the glycocoll may be either synthesized in the body or formed by the cleavage of other amino-acids. Thus A. I. Ringer<sup>4</sup> has

<sup>1</sup> Moll *Hofmeister's Beiträge*, iv, p. 563, 1904, vii, p. 311, 1906.

<sup>2</sup> For the literature, see Gibson and Banzhaf *Journ. of Exp. Med.*, xii, p. 411, 1910.

<sup>3</sup> Hammarsten-Mandel *Text-book of Physiological Chemistry*, p. 103, 1911.

<sup>4</sup> A. I. Ringer *This Journal*, x, p. 327, 1911.

found that goats have the power of eliminating hippuric acid containing much more glycocoll than would be found preformed in the proteins metabolized Epstein and Bookman<sup>5</sup> have also shown that after carbohydrate feeding, the production of glycocoll for the formation of hippuric acid can occur independently of the rest of the protein metabolism The possibility of the formation of glycocoll from other amino-acids, at least *in vivo*, is not excluded Osborne and Mendel<sup>6</sup> have just shown, also, that certain proteins, deficient in glycocoll, not only will maintain the normal waste and repair of the tissues, but will even serve for growth purposes as well

Recently Breinl,<sup>7</sup> extending the work of Cervello<sup>8</sup> on the quantitative alterations of the blood proteins after the administration of antipyrine, has described an increase in the serum globulin and a diminished albumin content of similar character to the changes reported during immunization He believes that he is dealing here with a transformation identical with Moll's conversion Breinl also points out that the glycocoll, which is present in the normal serum globulin, might be formed from the cleavage of the cystine of the serum albumin and would accordingly be obtained on the hydrolysis of the artificial serum globulin

Partly in view of this newer work on the blood changes after antipyrine and on the origin of glycocoll, and in part as a preliminary work for the study of the nature of the protein changes during immunization, it seemed worth while to take up once more this so-called transformation of serum albumin into serum globulin The changes which the albumin has undergone in Moll's conversion may most easily be followed by determining the distribution of the nitrogen according to Hausmann If the artificial globulin resembled the natural serum globulin in this respect, the protein could be hydrolyzed and glycocoll isolated if present

The partition of the nitrogen in Moll's artificial pseudoglobulin was accordingly determined As was expected, it was shown that the artificial pseudoglobulin (soluble in saturated sodium chloride

<sup>5</sup> Epstein and Bookman *This Journal*, x, p 353, 1911

<sup>6</sup> Osborne and Mendel *Bull of the Carnegie Inst of Washington*, D C , No 156, Part II

<sup>7</sup> Breinl *Arch f exp Path u Phar*, lxx, p 309, 1911

<sup>8</sup> Cervello *Ibid*, lxx, p 357, 1910

solution) differs from the serum albumin—so far as the nitrogen distribution is concerned—only in having a smaller *amid* nitrogen content. A determination on some normal serum globulin, precipitated at half saturation ammonium sulphate and dissolved in saturated sodium chloride solution, was made for comparison. The results indicate that the natural and artificial globulins are not identical.

A 2 per cent solution of crystallized and dialyzed serum albumin (from horse blood) was heated for an hour with an equal amount of 5% sodium carbonate solution as directed by Moll. The mixture was then precipitated with an equal volume of saturated ammonium sulphate solution. The precipitate was redissolved, again precipitated at half saturation, filtered and pressed out between filter papers. The artificial globulins were then dissolved in water, filtered through pulp, saturated with sodium chloride and filtered after standing for a few hours. The "globulin" was almost entirely soluble in the saturated sodium chloride solution. The filtrate was precipitated with acetic acid, filtered and pressed out between dry filter paper. It was then redissolved in water, filtered through pulp and coagulated by heat. The coagulum was collected on a Buchner funnel, washed until the filtrate no longer gave a sulphate or chloride reaction, was treated with hot alcohol and dried. A nitrogen determination gave 15.95 per cent by the Kjeldahl method.

The determinations of the nitrogen partition for the artificial pseudoglobulin are given as percentages of the total protein in the following table, along with a similar analysis on the normal serum albumin by Osborne and Harris<sup>9</sup>

	ARTIFICIAL PSEUDOGLOBULIN		SERUM ALBUMIN (OSBORNE AND HARRIS)
	I	II	
Amid N*	0.64	0.63	1.01
Non-basic N†	9.38	9.44	9.61
Basic N	5.77	5.88	5.30
Melanoid N	0.16	0.14	0.16

\* Denis. *This Journal* viii, p. 427, 1910.

† By difference.

<sup>9</sup> Osborne and Harris. *Journ. of the Amer. Chem. Soc.*, xxv, p. 323, 1903.



As seen in the table, there are no noteworthy differences in the nitrogen partition, except the low amid nitrogen. The diamino nitrogen is slightly higher than Osborne and Harris' figure, but the method for this determination is hardly exactly quantitative, and pronounced differences only can be considered.

In the following table are given the results of the analyses for the nitrogen partition in the saturated sodium chloride soluble normal serum globulin (from horse serum). The preparation contained 15.60 per cent of nitrogen. The results show a higher melanoid nitrogen and the characteristically low basic nitrogen. The figures in these respects are essentially different from those obtained for the artificial pseudoglobulin.

	NORMAL PSEUDOGLOBULIN	
	I	II
Amid N	0.95	1.04
Non-basic N	10.84	10.60
Basic N	3.38	3.51
Melanoid N	0.43	0.45

It would seem, then, that Moll's artificial serum globulin is to be regarded simply as an intermediate stage in the formation of the alkali metaprotein.

# INORGANIC PHOSPHORUS IN PLANT SUBSTANCES AN IMPROVED METHOD OF ESTIMATION

By R C COLLISON

*(From the Department of Nutrition of the Ohio Agricultural Experiment  
Station, Wooster, Ohio )*

(Received for publication, May 16, 1912 )

In the synthesis of organic phosphorus compounds by the plant and in their storage in the seed as reserve food the inorganic phosphates play an important part. The phosphorus used in this synthetic process is taken into the plant in inorganic form and used in the building up of complex organic phosphorus compounds such as lecithins, phospho- and nucleo-proteins and salts of phytic acid.

It is believed by some investigators that unmodified plant tissue and a large number of the seeds contain practically no phosphorus in inorganic combination. Since inorganic phosphate is the starting point in the synthesis of organic phosphorus compounds and since there exist in the plant enzymes which have the property of splitting off inorganic phosphorus from such compounds, it would seem probable that at least an appreciable quantity of phosphorus in inorganic form might be found in such plant tissues and seeds.

At any rate, such a supposition has been well sustained in a previous paper from this laboratory by E B Forbes, A Lehmann, R C Collison and A C Whittier, in which was included the original acid alcohol method of inorganic phosphorus estimation.

Methods of estimating inorganic phosphorus in plant substances usually begin with some means of acid extraction of the material with subsequent separation of the phosphates from the extract. Some of the proposed methods may be questioned on the ground of mechanical imperfection and also because they do not take into sufficient consideration the influence of organic matter on the precipitation of phosphates with the common precipitating agents. This influence on precipitation was investigated two years ago in

connection with the above mentioned paper<sup>1</sup> Dilute acid extracts of plant substances contain, besides inorganic salts, proteins, carbohydrates and, in the case of cereals, salts of phytic acid The proteins and carbohydrates both inhibit the formation of ammonium-phospho-molybdate and the salts of phytic acid, especially, have the property of suppressing the formation of this precipitate in a marked degree This influence of phytin was reported upon in our previous publication That it is no small factor contributing to the accuracy of the final results can be readily appreciated from the results below, which are quoted from the previous paper

TABLE 1  
*Influence of phytin on the precipitation of phosphorus*

GRAMS PHYTIN	GRAMS PHOSPHATE RECOVERED
1 0000	0 0000
0 5000	0 0260
0 4000	0 0329
0 3000	0 0371
0 0000	0 0420

Phytin is only one of the substances present in acid extracts of seeds which may have a retarding or inhibiting influence on the precipitation of phosphorus

These results, it is believed, indicate very clearly that some failures to demonstrate the presence of inorganic phosphorus in seeds could have been due to imperfections in the analytical methods

There is no absolute standard with which we can compare results by different methods The only standard we have for comparison is one established by the addition of a known quantity of pure phosphate to a plant substance together with the constancy of analytical results Complete recovery of the phosphorus added and a constant result for inorganic phosphorus without such addition, would then constitute a fair test of any given method

The difficulties in precipitation above noted, have been largely overcome in the original acid-alcohol method of this department<sup>2</sup> A brief description of this method is here given

<sup>1</sup> Bulletin 215, Ohio Agr Exp Sta, April 1910

<sup>2</sup> *Loc cit*

Commercial phytin was dissolved in 0.2 per cent nitric acid and pure sodium phosphate, equivalent to 0.0420 gram of magnesium pyrophosphate, was added. Acid molybdate solution in excess was then added and the solutions digested at 60° C for one hour. The volume of the solutions was about 200 cc.

Ten grams of the substance were extracted with 300 cc of 0.2 per cent hydrochloric acid for three hours and 250 cc of the filtered extract were precipitated with magnesia mixture and made strongly ammoniacal. After twelve hours the precipitate was filtered off and washed with ammonia and finally with alcohol. The dried paper with the precipitate was thoroughly shaken with a measured volume of acid alcohol, the mixture filtered, an aliquot of the filtrate evaporated and phosphorus determined in the residue.

This method avoids the necessity of precipitating phosphorus in the presence of proteins and salts of phytic acid. It has now been in use for two years in this laboratory and has given more satisfactory results than any method previously used.

In all the plant substances examined from time to time, appreciable quantities of inorganic phosphorus have been found by this method. A few results are here given for illustration.

TABLE 2  
*Total and inorganic phosphorus in a few plant substances*

SUBSTANCE	TOTAL P	INORGANIC P
	<i>per cent</i>	<i>per cent</i>
Oats, grain	0.397	0.060
Wheat, grain	0.394	0.036
Corn, grain	0.266	0.041
Soy beans	0.547	0.054
Cow peas	0.445	0.056
Rice polish	0.600	0.027
Alfalfa hay	0.230	0.136
Blue grass	0.256	0.158

With substances giving extracts low in organic material, as is the case with the rough feeds, such as the hays, and also with many other substances the method gives consistent results. But with substances giving extracts high in organic material, this method may cause, in some cases, high results due to great difficulty in filtering the extracts, the prolonged standing probably causing decomposition of organic phosphorus compounds, and in other cases, low results due to the influence of organic substances on precipitation.

of magnesium-ammonium-phosphate These difficulties are met with especially in the extracts of leguminous seeds which are in general extremely difficult to filter

With the purpose of eliminating these possible sources of objection, the author proposes a modified and improved method which retains the essential point in the original method, namely the acid alcohol separation of phosphates from phytates, but differs from the original in the omission of the preliminary acid water extraction and magnesia mixture precipitation, the acid alcohol separation being performed on the fresh substance direct, and differing also by such arrangement of details as permits of the saving of the alcohol

As was demonstrated in the publication of the original acid alcohol method, cold acid alcohol (94 per cent alcohol containing 0.2 per cent hydrochloric or nitric acid) has the property of dissolving the common phosphates which are insoluble in neutral or slightly alkaline alcohol and of separating them from phytates, which are insoluble in this reagent Most proteins, nucleic acid from yeast, and carbohydrates appear for the most part to be insoluble Taking these facts into consideration, a direct acid alcohol extraction is proposed Such an extraction would insure solution of inorganic phosphates, which could be filtered from the undissolved organic phosphorus compounds in the residue

For the investigation of this improved method, three plant substances were selected

- 1 Soy beans, on account of their high percentage of soluble protein and also because dilute acid-water extracts of the ground beans, as well as the precipitated extracts, are extremely difficult to filter

- 2 Rice polish, on account of its rather high content of phytin

- 3 Corn germ meal, on account of its content of nucleic acid phosphorus

The improved method of procedure, in detail, is as follows

A 10 gram sample of the substance, very finely ground, is placed in a 400 cc flask and covered with exactly 300 cc of 94 to 96 per cent phosphorus free alcohol, which contains 0.2 per cent of hydrochloric acid (calculated from the per cent HCl in the concentrated acid) The flask is shaken at intervals of five minutes for three hours The extract so obtained is then filtered through dry, double 11 cm filters into dry flasks No suction is necessary An aliquot of 250 cc of this filtrate is placed in a 400 cc beaker and made just alkaline to litmus paper with ammonia A slight excess of

ammonia does no harm. The solutions are allowed to stand from eight to twelve hours or over night and then filtered through double 11 cm filters, care being taken to decant the clear liquid as far as possible. The precipitate is then transferred to the filter and washed with 94 to 96 per cent alcohol which has been made just ammoniacal. In transferring the precipitate some of the material may stick very tenaciously to the beaker. In this case, after cleaning the beaker fairly well, add five drops of hydrochloric acid to the beaker, rub out the latter with a rubber tipped rod, add 10 cc of alcohol and then make just alkaline with ammonia and transfer this last portion to the filter. In this way the last traces of the precipitate can be easily removed. After washing several times, the inner filter with the precipitate is spread out and allowed to dry completely. It is then transferred to an Erlenmeyer flask containing exactly 100 cc of 0.5 per cent nitric acid in water (calculated from the per cent  $\text{HNO}_3$  in the concentrated acid). The flask is stoppered and the contents thoroughly shaken until the paper and precipitate are broken up. It is best to let it stand for some hours. The material in the flask is then filtered through dry, double 11 cm filters into dry beakers and exactly 75 cc of the filtrate precipitated with 50 cc of official acid molybdate solution in the usual way, 10 grams of ammonium nitrate and two hours digestion at  $60^\circ\text{C}$  are usually sufficient. The final result represents the amount of inorganic phosphorus in 6.25 grams of the original sample.

It is advisable to reprecipitate the pyrophosphate, if the final solutions are highly colored, which is sometimes the case with some of the rough feeds, as the hay. In the case of such substances, which are relatively high in inorganic phosphorus, a smaller sample may be taken, 3 to 6 grams. In using this method with substances which are tenacious and gummy and which do not break up readily in acid alcohol, as is true of dried fruits and other substances containing considerable sugar, the same may be worked up with sand and a definite quantity of water, 15 to 20 cc are usually sufficient. This may be done in a mortar and the material washed out into the flask with acid alcohol, care being taken to use the correct volume, namely 300 cc minus the quantity of water used.

This method deflocculates substances the most refractive in this regard.

Three sets of determinations were made by this method on soy beans, rice polish and corn germ. In each case determinations were made with and without the addition of a known amount of pure phosphate, in an endeavor to recover the latter completely.

The first two sets of results were slightly low, due to insufficient time given for precipitation, since the filtered extracts were made ammoniacal and filtered immediately. Following the detailed method as stated, allowing the extracts to stand after precipitation the specified twelve hours, excellent results were obtained as shown by the following table.

TABLE 3

*Recovery of added phosphorus by improved method*

SUBSTANCE	WITHOUT PHOSPHATE	WITH PHOSPHATE 0.0037 GRAM P	THEORETICAL RESULTS	INORGANIC P
	gram	gram	gram	per cent
Soy beans	0.0015	0.0053	0.0052	0.024
Rice polish	0.0012	0.0048	0.0048	0.019
Corn germ	0.0018	0.0053	0.0054	0.029

The figures are grams of phosphorus (element) and are averages of three determinations. The first column of figures represents the quantity of inorganic phosphorus found in the material itself. Column two represents the amount found after the addition of pure phosphate in known quantity, and column three, the results which should have been obtained after adding pure phosphate, assuming that the figures in column one are the correct ones for inorganic phosphorus. For example, the inorganic phosphorus in 6.25 grams of soy beans amounted to 0.0015 gram of phosphorus. To every 6.25 grams of sample was added pure sodium phosphate equivalent to 0.0037 gram of phosphorus. Thus if the added phos-

TABLE 4

*Inorganic phosphorus by improved method with and without pure phosphate*

SUBSTANCE	WITHOUT PHOSPHATE	WITH PHOSPHATE 0.0037 GRAM P	WITH PHOSPHATE REPRECIPITATED	THEORETICAL RESULTS	INORGANIC P
	gram	gram	gram	gram	per cent
Cow peas	0.0014	0.0053	0.0051	0.0051	0.023
Oil meal	0.0017	0.0057	0.0053	0.0054	0.027
Corn meal	0.0016	0.0056	0.0051	0.0053	0.025
Wheat, grain	0.0008	0.0046	0.0044	0.0045	0.012
Wheat, bran	0.0021	0.0063	0.0060	0.0058	0.034
Wheat, germ	0.0025	0.0068	0.0064	0.0062	0.040
Clover hay	0.0043	0.0085	0.0082	0.0080	0.070
Timothy hay	0.0030	0.0068	0.0067	0.0067	0.047
Oats, grain	0.0026	0.0067	0.0064	0.0063	0.041
Cottonseed meal	0.0014	0.0055	0.0053	0.0051	0.023
Rice polish	0.0012	0.0050	0.0049	0.0049	0.019
Corn germ	0.0018	0.0055	0.0055	0.0055	0.029
Soy beans	0.0015	0.0053	0.0052	0.0052	0.024

phorus was completely recovered, the result should have been  $0.0015 + 0.0037 = 0.0052$  gram of phosphorus for the theoretical

These results check with the theoretical as closely as could be desired, indicating complete recovery of the phosphate

In table 4 are given similar results on a more extended scale. The pyrophosphate was reprecipitated, the results being given in column three.

The figures represent grams of phosphorus (element). Here again the results agree well with the theoretical in every case. The extracts of substances which, by the original method, filter with great difficulty, by the improved method filter with great ease, as well as the solutions after precipitating by neutralization.

In order to make the comparison complete a similar series of determinations was made by the original method, on the same substances, with and without phosphate added.

TABLE 5

*Results by original method      Comparison of original and improved methods*

SUBSTANCE	WITHOUT PHOSPHATE	WITH PHOSPHATE 0.0081 GRAM P	THEORETICAL RESULTS	INORGANIC P	
				Original Method	Improved Method
	gram	gram	gram	per cent	per cent
Cow peas	0.0013	0.0068	0.0094	0.020	0.023
Oil meal	*	*	*	*	0.027
Corn meal	0.0014	0.0092	0.0095	0.022	0.025
Wheat, grain	0.0006	0.0074	0.0087	0.009	0.012
Wheat, bran	0.0037	0.0098	0.0118	0.059	0.034
Wheat, germ	0.0022	0.0090	0.0103	0.035	0.040
Clover hay	0.0037	0.0088	0.0118	0.059	0.070
Timothy hay	0.0026	0.0105	0.0107	0.042	0.047
Oats, grain	0.0034	0.0110	0.0115	0.055	0.041
Cottonseed meal	0.0048	0.0124	0.0129	0.076	0.023
Rice polish	0.0016	0.0087	0.0097	0.025	0.019
Corn germ	0.0018	0.0097	0.0099	0.029	0.029
Soy beans	0.0010	0.0032	0.0047	0.015	0.024

Unfilterable

The results in the first three columns are expressed in grams of phosphorus (element). The inorganic phosphorus was recovered in five cases, namely, corn meal, timothy hay, oats, cottonseed meal and corn germ. It is interesting in this connection that all



five are substances which do not give a large quantity of organic material on extraction. On the other hand, cow peas, wheat, wheat bran, wheat germ, clover hay, rice polish and soy beans are all plant substances which yield a large quantity of soluble organic matter on extraction, which has evidently had its effect in preventing complete precipitation, since the phosphate added was not entirely recovered in these substances.

Hydrolysis has possibly been a factor contributing to the variations in the results by the two methods, especially in the case of cottonseed meal and wheat bran. The higher results in these two substances may indicate the splitting off of inorganic phosphorus from organic compounds. This factor could have considerable bearing on results by the original method in which extraction and precipitation are made in a water solution. On the other hand, in the improved method this factor would not have special significance, since extraction and filtration are made in alcohol, and the presence of strong alcohol, practically throughout the process, tends to prevent enzyme action and bacterial decomposition.

As a factor in causing low results may be mentioned the property of some organic bodies to combine with inorganic salts in water solution and also the property of such bodies of inhibiting the precipitation of phosphorus, either chemically or mechanically. Phytin, proteins and carbohydrates seem to have these properties, some in a marked degree.

The chief considerations which recommend the improved acid-alcohol method may be stated as follows:

- 1 Alcohol extraction prevents enzyme and bacterial decomposition of organic phosphorus compounds.

- 2 Most organic phosphorus compounds seem to be practically insoluble in the solvent.

- 3 Filtration of the extracts of even the most difficultly filterable materials is very rapid.

- 4 Recovery of the alcohol used is possible.

- 5 A considerable saving of time over the old method is secured.

- 6 Results on all the substances examined seem to be constant and, in all cases, added inorganic phosphorus was completely recovered.

The author wishes to express thanks to Dr. E. B. Forbes for his courtesy in making this investigation possible.

## EXPERIMENTAL STUDIES ON CREATINE AND CREATININE

### IV THE ESTIMATION OF CREATINE IN THE PRESENCE OF SUGAR

By WILLIAM C ROSE

*(From the Laboratory of Physiological Chemistry, School of Medicine, University of Pennsylvania )*

(Received for publication, May 20, 1912 )

In the course of studies of certain phases of metabolism during diabetes, it was observed that the determination of creatine by the Benedict-Myers modification of the Folin method yields results much too high. In the presence of sugar, heating in the autoclave with normal hydrochloric acid oxidizes the sugar with the production of dark brown compounds which seriously interfere with the accuracy of the method. These substances not only change the character of the color, giving it a brownish tint difficult to match with the bichromate solution, but they also greatly increase the color intensity, i e, the sugar decomposition products themselves give the creatinine reaction. When pure solutions of dextrose are heated in the autoclave with equal volumes of normal hydrochloric acid, and the resulting mixtures treated with picric acid and alkali, intensely red solutions result. Moreover, the brown sediment which separates during the autoclave heating of sugar-containing urines gives the Jaffé creatinine test when filtered off, washed, and treated with picric acid and alkali.

A single series of determinations on a normal human urine to which increasing quantities of dextrose were added, will suffice to indicate the magnitude of the analytical errors. In this table, as in all succeeding ones, the quantities of creatinine are calculated from the averages of six to ten readings.

It is obvious that determinations by this method in the presence of sugar give readings decidedly too low (corresponding to too

TABLE I

*The influence of dextrose on the estimation of creatine by the HCl-autoclave method*

NUMBER	SUGAR ADDED PER 10 CC URINE	READINGS	TOTAL CREATININE
	gram	mm	mgm
1	0 0	10 1	8 02
2	0 1	8 0	10 12
3	0 2	6 5	12 45
4	0 4	6 1	13 27
5	0 7	6 2	13 05

much total creatinine) Frequently the creatine findings are 10 to 50 per cent too large and, when considerable sugar and relatively small amounts of total creatinine are present, the creatine values may even be 100 per cent too large. In fact it is surprising that the errors resulting from creatine estimations in diabetic urines have not been emphasized to a greater extent. Dreibholz<sup>1</sup> called attention to the difficulty in matching colors after heating with hydrochloric acid, but apparently overlooked the fact that the decomposition products of sugar give the Jaffé reaction.

To overcome the errors incident to the estimation, Dreibholz attempted to remove the sugar from the urines by fermentation, but reported that the creatine-creatinine figures were then far too low, presumably owing to the destruction of creatine and creatinine by enzymes (creatase and creatinase) present in the yeast. So far as the writer is aware, these experiments have never been verified but even though creatase and creatinase were absent from yeast, fermentation would not be a desirable method of overcoming the difficulties in creatine estimation. The possibility of bacterial decomposition of creatine and creatinine in the fermenting urines, and the necessary delay of twenty-four hours in obtaining the analytical results, would render the procedure unsatisfactory.

In order to overcome the errors in the method, numerous attempts have been made to remove the interfering substances by decolorization with lead salts, colloidal iron hydroxide, etc., but

<sup>1</sup> Dreibholz *Inaug. Diss. Greifswald*, 1908

all attempts have been unsuccessful, because of a loss of creatinine in the process<sup>2</sup> The only procedure that promised success was to obtain an acid which would quantitatively convert creatine into its anhydride and still would not caramelize sugar during the autoclave heating After experiments with several weak acids, a satisfactory one was found in phosphoric acid Heating in the autoclave with dilute solutions of phosphoric acid produces no perceptible oxidation of dextrose, even when the latter is present in relatively large concentrations (8 to 10 per cent) The sugar solutions are water-clear when removed from the autoclave Moreover, creatine in all concentrations that occur in urines under physiological and pathological conditions, is quantitatively converted into creatinine by heating with twice the volume of 3 per cent phosphoric acid, for a period of thirty minutes at a temperature of 117° to 120°C

Phosphoric acid has an additional advantage over hydrochloric acid in that it produces much less oxidation of the urinary pigments The darkening of the color of urine by heating with hydrochloric acid, and the possibility of analytical errors resulting therefrom, has been alluded to by several investigators<sup>3</sup> Benedict<sup>4</sup> has recently emphasized the possibility of errors that may ensue from pigment oxidation He found that the precipitate which separates during the heating of normal urine with hydrochloric acid, gives the creatinine reaction, and to overcome this consequence he suggests the addition of granulated lead to the urine-hydrochloric acid mixture and evaporation to dryness over the free flame The creatine is quantitatively converted into creatinine during the evaporation and the small amount of hydrogen liberated from the acid by the lead prevents pigment oxidation It has been the writer's experience that while this method gives beautiful results in sugar-free urines, the amount of hydrogen liberated is not sufficient to prevent sugar oxidation in diabetic urines In fact, if the urine contains sugar, direct evaporation over the free flame produces much more coloration than does the autoclave method of Benedict and Myers

<sup>2</sup> Dreibholz *loc cit*

<sup>3</sup> Weber *Arch exp Path u Pharm*, lvm, p 93, 1907, *Dorner Zeitschr f physiol Chem*, lvi, p 227, 1907

<sup>4</sup> Benedict *Proceedings Amer Soc Biol Chem*, Baltimore, Dec, 1911

The phosphoric acid method is now used by the writer in all human urines, either normal or diabetic, and has been found to give very satisfactory results. The procedure is as follows. To 10 cc of urine are added 20 cc of 3 per cent phosphoric acid, and the mixture is heated for thirty minutes in the autoclave at a temperature of  $117^{\circ}$  to  $120^{\circ}\text{C}$ . The solution is then cooled, neutralized, treated with sodium hydroxide and picric acid as in the Folin procedure, and allowed to stand for five minutes. At the expiration of this time, it is diluted to a volume sufficient to make the readings in the colorimeter lie within the range of 7 and 12 mm. In the presence of sugar it is advisable to let the colored solution stand for three or four minutes after dilution before making the readings. Dextrose itself tends to give a faint creatinine reaction after standing five to ten minutes with the picric acid and alkali. Fortunately, however, this color rapidly fades on dilution, and entirely disappears within three or four minutes. This delay causes no change in the intensity of the color due to creatinine. It is necessary to avoid heating above  $120^{\circ}\text{C}$  and the use of acid stronger than 3 per cent. At higher temperatures and with acid of greater concentration, creatinine is partially destroyed.

The above modification has been used in several hundred creatine estimations on human and rabbits' urines. Its accuracy has been thoroughly tested with diabetic and normal urines, with and without the addition of sugar, with and without the addition of creatine and creatinine, and in pure solutions of creatine and creatinine with and without the addition of sugar. With widely varying amounts of creatine and creatinine, and in the presence of dextrose in concentrations up to 8 per cent, it gives results as near theoretical as can be expected for a colorimetric method in which exactness necessarily depends in part upon the accuracy of the eye. Typical series of determinations on normal and diabetic urines are shown in Tables II to V. The readings tabulated are for 10 cc amounts of urine diluted to volumes of 500 cc. When the readings came below 7 or above 12 mm, the greater dilutions were used, or estimations were repeated with more urine. The corresponding readings for the arbitrary conditions of 10 cc diluted to 500 cc were then calculated.

For reasons as yet undetermined, the phosphoric acid modification does not give satisfactory results with dog's urine. Fre-

TABLE II  
Normal human urine

NUMBER	DETERMINATION	FOR 10 CC URINE	
		Readings	Creatinine
		mm	mgm
1	Preformed creatinine,	13 6	5 96
2	Total creatinine by HCl-autoclave method	13 2	6 14
3	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	13 4	6 04
4	Same as 3 + 0 6 gm dextrose per 10 cc	13 2	6 14
	<i>In 100 cc of the same urine were dissolved 34 5 mgm of creatine, corresponding to 3 mgm of creatinine per 10 cc of urine</i>		
5	Total creatinine by HCl-autoclave method	9 0	9 00
6	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	8 9	9 10
7	Same as 6 + 0 6 gm dextrose per 10 cc	9 0	9 00
	<i>In a second portion of the original urine were dissolved 116 mgm of creatine per 100 cc, corresponding to 10 mgm of creatinine per 10 cc</i>		
8	Total creatinine by HCl-autoclave method	5 0	16 19
9	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	4 9	16 52
10	Same as 9 + 0 6 gram dextrose per 10 cc	5 0	16 19
11	Same as 9 + 0 8 gram dextrose per 10 cc	5 0	16 19

quently the result for the total creatinine is considerably lower than for the preformed. Canine urine, as is well known, contains several products not present in the urine of man or of rabbits. It is possible that the presence of kynurenic acid or of ethyl sulphide may be the disturbing factor, though it is difficult to understand why similar results are not obtained with hydrochloric acid. The matter is now under investigation, and it is hoped that in the near future the difficulties in obtaining accurate results in the urine of dogs may be overcome.

Krause<sup>5</sup> has recently reported that the presence of acetoacetic acid causes a significant increase in the intensity of the color produced by picric acid and sodium hydroxide, thereby leading to results too high for the preformed creatinine. In consequence

<sup>5</sup> Krause *Quart Journ of exp Physiol*, III, p 298, 1910

TABLE III

*Normal human urine*

NUMBER	DETERMINATION	• FOR 10 cc URINE	
		Readings	Creatinine
		mm	mgm
1	Preformed creatinine	7 6	10 65
2	Total creatinine by HCl-autoclave method	6 5	12 45
3	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	6 6	12 27
4	Same as 3 + 0.4 gram dextrose per 10 cc	6 5	12 45
5	Same as 3 + 0.8 gram dextrose per 10 cc	6 6	12 27
	<i>Creatine added to urine—3.48 mgm per 10 cc, corresponding to 3 mgm creatinine</i>		
6	Total creatinine by HCl-autoclave method	5 1	15 87
7	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	5 2	15 57
8	Same as 7 + 0.6 gram dextrose per 10 cc	5 1	15 87
	<i>Creatine added to original urine—11.6 mgm per 10 cc, corresponding to 10 mgm creatinine</i>		
9	Total creatinine by HCl-autoclave method	3 5	23 14
10	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	3 6	22 50
11	Same as 10 + 0.6 gram dextrose per 10 cc	3 6	22 50

of this, the creatine values are, according to him, correspondingly too low. He further states that the presence of acetone has no influence on the creatine-creatinine estimations, owing to the rapidity with which the color produced by acetone fades.

To test the influence of acetoacetic acid and acetone, a series of experiments were performed in which varying amounts of these substances were added to normal urine, and the creatinine subsequently estimated. Acetoacetic acid, in quantities not exceeding 0.25 gram per 100 cc of urine, produced no alteration in the readings, provided the tests were allowed to stand three or four minutes after dilution before making the readings. During this time the color due to the acetoacetic acid entirely fades. The result in one such test was as follows: To 100 cc of normal urine, which contained 9.42 mgms of preformed creatinine per 10 cc, was added the amount of ethyl acetoacetic ester equivalent to 0.25 gram of free acid. Creatinine was then estimated, three

TABLE IV  
Mild diabetes—0.8 per cent dextrose

NUMBER	DETERMINATION	FOR 10 cc URINE	
		Readings	Creatinine
		mm	mgm
1	Preformed creatinine	8.4	9.64
2	Total creatinine by HCl-autoclave method	7.9	10.25*
3	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	8.4	9.64
	<i>Creatine added to urine—4.64 mgm per 10 cc, corresponding to 4 mgm creatinine</i>		
4	Total creatinine by HCl-autoclave method	5.6	14.46*
5	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	5.9	13.72
	<i>Creatine added to urine—11.6 mgm per 10 cc, corresponding to 10 mgm creatinine</i>		
6	Total creatine by HCl-autoclave method	4.1	19.75
7	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	4.2	19.28

\* Note higher results with the HCl method in the presence of dextrose

TABLE V  
Severe diabetes—2 per cent dextrose, carbohydrate-free diet

NUMBER	DETERMINATION	FOR 10 cc URINE	
		Readings	Creatinine
		mm	mgm
1	Preformed creatinine	13.4	6.04
2	Total creatinine by HCl-autoclave method	9.6	8.44*
3	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	11.2	7.23
	<i>Creatine added to urine—3.48 mgm per 10 cc, corresponding to 3 mgm creatinine</i>		
4	Total creatinine by HCl-autoclave method	7.1	11.40*
5	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	8.0	10.12
6	Same as 5 + 0.5 gm dextrose per 10 cc	8.1	10.00
	<i>Creatine added to original urine—11.6 mgm per 10 cc, corresponding to 10.0 mgm of creatinine</i>		
7	Total creatinine by HCl-autoclave method	4.4	18.40
8	Total creatinine by H <sub>3</sub> PO <sub>4</sub> -autoclave method	4.7	17.22
9	Same as 8 + 0.5 gram dextrose per 10 cc	4.7	17.22

\* Note the effect of dextrose on the estimations by the HCl method



minutes being allowed for fading. The readings showed the presence of 9.31 mgms of creatine per 10 cc. Manifestly, such differences are well within the limits of accuracy of the method. In larger concentrations acetoacetic acid tends to increase the creatinine values. It is rare, however, that a specimen of diabetic urine is obtained which contains more than 0.25 per cent of acetoacetic acid.

Acetone in all concentrations is without influence on the creatinine readings.

It is evident, therefore, that by the use of phosphoric acid instead of hydrochloric acid, and by allowing the diluted solutions to stand for a few minutes before making the readings, just as accurate estimations of creatine and creatinine may be made in diabetic as in normal urines.

# FEEDING EXPERIMENTS WITH FAT-FREE FOOD MIXTURES <sup>1</sup>

By THOMAS B OSBORNE AND LAFAYETTE B MENDEL

WITH THE COÖPERATION OF EDNA L FERRY

(From the Laboratory of the Connecticut Agricultural Experiment Station,  
and the Sheffield Laboratory of Physiological Chemistry in Yale University,  
New Haven, Connecticut)

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Proteins (or their constituent amino-acids<sup>2</sup>) and carbohydrates in some physiologically available form are indispensable components of the diet. If either of them are entirely eliminated from the food intake nutrition sooner or later becomes notably defective, however liberal the energy values of the remaining nutriment may be. The ketonuria and acidosis which appear along with other phenomena when carbohydrate utilization completely fails are familiar, and the need of protein-nitrogen is one of the fundamental postulates of physiology.

With respect to the actual requirement of fat on the part of the healthy organism there is at present almost no definite information available. Fats are, of course, commonly found present in greater or lesser abundance in every dietary, but to what extent they represent an indispensable need of the animal remains to be learned. The reason why this apparently fundamental question in nutrition has not been answered before is presumably attributable to the experimental difficulties inherent in its solution. Fats or fat-like substances are present to some extent in the majority of the familiar food materials, from which they can be completely removed only with the expenditure of considerable effort and care, and the at-

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D C

<sup>2</sup> Cf Abderhalden, E *Zeitschr f physiol Chem*, lxxvii, p 22, 1912

tempts to maintain animals on artificially prepared mixtures of isolated food substances have, until lately, met with little success.<sup>3</sup>

Associated with this problem is the possible significance of that heterogeneous group of substances, resembling the fats in certain physical properties, found with them in nature, and currently designated as "lipoids." As representatives of this category the phosphatides, and cholesterols, like the inorganic salts, are found present in some quantity in every active cell.<sup>4</sup> This fact of itself strongly suggests for them some preëminent biochemical importance, but it by no means involves the necessity of their being furnished as such to the organism. It is easily conceivable that the so-called lipoids can be synthesized *de novo* by the animal tissues as they unquestionably are by plant cells. The fact that the food supply of growing organisms, viz., milk or egg components, furnishes phosphatides and cholesterol preformed speaks only by indirect suggestion regarding the absolute need of these compounds in the diet. Finally, the fact that in tissues, the "lipoids" are so closely associated with the true fats, i. e., glycerides of fatty acids, by no means proves that the biological importance of the two groups is comparable or their significance as dietary constituents the same.

The question of the rôle of fats as indispensable factors in the diet has been approached by Stepp.<sup>5</sup> In attempting to ascertain whether animals are dependent upon their food supply for lipoids or can furnish them by synthesis like plants, he fed materials extracted with ether and alcohol to mice and observed the effect on the nutritive equilibrium of the animals. Obviously this method of preparing the food eliminated true fats from the diet at the same time. Stepp's observations and conclusions deserve to be carefully examined in connection with the problem at hand. He noted that without exception his mice succumbed in a few weeks when offered otherwise adequate food mixtures that had been thoroughly extracted. The deduction is made that the nutritive failure is due

<sup>3</sup> A discussion of earlier attempts in this direction will be found in our monograph *Feeding Experiments with Isolated Food-Substances*, Carnegie Institution of Washington, Publication 156, Parts I and II, 1911.

<sup>4</sup> For a general description of the so-called lipoids, their occurrence and possible biochemical significance, see Bang *Ergeb d Physiol*, vi, p 131, 1907, viii, p 463, 1909.

<sup>5</sup> Stepp *Biochem Zeitschr*, xxii, p 452, 1909, *Verhandl Kongresses f inn Med*, xxviii, p 324, 1911, *Zeitschr f Biol*, lvi, p 135, 1911.

to the lack of certain "lipoid" substances, because the addition of alcohol-ether extracts of materials known to be rich in this type of compound sufficed to keep the animals alive. The lacking substance is assumed not to be inorganic, since the addition of the ash of the lipid extracts made from the food material failed to maintain the mice. Furthermore—and this calls for emphasis here—the sustaining component is asserted not to be ordinary fat inasmuch as the addition of so typical a fat as butter failed to replace the missing life-sustaining factor. The latter was found to be extractable from skimmed milk rather than from the cream fraction. Quoting Stepp

The life-sustaining alcohol-ether-soluble food components, in the absence of which mice regularly succumb, are not *fats*. This is shown, aside from the experiments in which butter was fed, by the following experiment. On a diet of extracted foods to which tripalmitin, tristearin and triolein are added, all the animals die precisely as on the extracted food alone. That lecithin (Merck) and cholesterol do not represent the sole lipoids essential to life is shown by the experiment of adding them to the extracted food: all of the animals died.<sup>6</sup>

It is rather difficult to believe that skimmed milk, at best very deficient in ether-alcohol soluble components, should contain an eminently important lipid in any adequate amount while other materials, like butter, which must contain some compounds of this type are inadequate. However, certain of these lipoids are doubtless highly sensitive to chemical change, so that it is conceivable that they lose their physiological potency through chemical manipulation. Furthermore the recent experiences with beri-beri and other forms of peripheral neuritis have emphasized how small may be the actual amount of a specific substance which determines proper physiological functioning.<sup>7</sup>

Stepp's experiments on mice by no means solve the question which we have raised at the outset with regard to the necessity of *fats* in the diet. They furnish no evidence that the "lipoid" mixtures which he employed to maintain or resuscitate his animals were actually free from true fats, though the quantities in some cases (such as the experiments with extract of skimmed milk) must at

<sup>6</sup> Stepp *Zeitschr f Biol*, lvii, p 170, 1911

<sup>7</sup> Cf Funk *Journ of Physiol*, xliii, p 395, 1911

best have been exceedingly small. Other occasional experiments in the literature<sup>8</sup> are of too brief duration to settle the point.

Employing the methods which were adopted in our earlier feeding experiments with isolated food substances<sup>9</sup> we have succeeded in inducing a normal rate of growth in white rats with dietaries devoid of fat throughout almost the entire period during which growth ordinarily continues.<sup>10</sup> The foods were prepared by mixing carefully isolated and purified proteins with starch, sugar and "protein-free milk,"<sup>11</sup> the latter having first been thoroughly extracted with ether. The starch was stirred with water, heated until the grains were ruptured and then the other ingredients thoroughly mixed with the starch paste, and afterwards dried in a current of hot air until thin cakes of desiccated food were obtained. These were then fed, along with the water, to the rats kept in the metabolism cages devised for these studies.<sup>12</sup> Although the foods may certainly be designated as fat-free, it is perhaps not permissible to speak of them as lipid-free, for according to the current definition, the so-called "lipoids" include substances soluble in hot alcohol which may not dissolve in ether. None of our isolated food materials were subjected to extraction with hot alcohol. Undoubtedly such treatment would remove other substances as well as lipoids from such a mixture as the "protein-free milk." This fact deserves to be emphasized, as does the necessity of conducting ether extractions under appropriate conditions. When, for example, a specimen of air-dry "protein-free milk" was extracted with ordinary ether it

<sup>8</sup> Cf. Lummert *Pflüger's Archiv*, lxxi, p. 176, 1898.

<sup>9</sup> Cf. Osborne, T. B., and L. B. Mendel *Feeding Experiments with Isolated Food-Substances*, Carnegie Institution of Washington, Publication 156, Parts I and II, 1911.

<sup>10</sup> In these, as in all our other experiments in which very young animals exhibited a normal rate of growth on mixtures of isolated food-substances, we have not yet succeeded in bringing the animals to their maximum normal size on the dietaries employed. This failure to attain complete growth involves some factor in nutrition other than the fat and is at present under investigation.

<sup>11</sup> For the character of this product cf. Osborne, T. B., and L. B. Mendel *Feeding Experiments with Isolated Food-Substances*, Carnegie Institution of Washington, Publication 156, Parts I and II, 1911, and *Science*, xxxiv, p. 722, 1911.

<sup>12</sup> Cf. Osborne, T. B., and L. B. Mendel *Zeitschr. f. biolog. Technik und Methodik*, 1912.

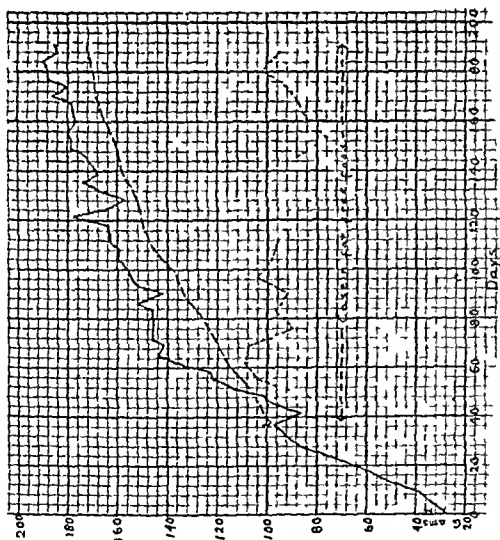


CHART 1 Rat 383, ♀, CHART 2 Rat 390, ♂ The food during the ensein fat free period had the following percentage composition

Casein	22
Sucrose	20
Starch	30
Protein free milk	8
	100

During the first part of the time 5 per cent of agar agar replaced an equal weight of starch and after the sixty fourth day the protein free milk was used without special extraction with ether

yielded over 2 per cent of extract, but the same product carefully dried in hydrogen and extracted with anhydrous ether yielded only 0.13 per cent of ether extract, which was not increased when an alkali solution of the substance was shaken out with benzine and ether according to the method commonly applied to milk powders. Actual extraction of the foods used by us yielded not more than an insignificant trace of ether extract.

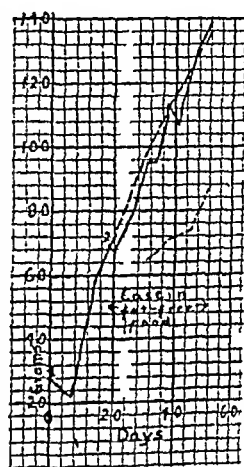
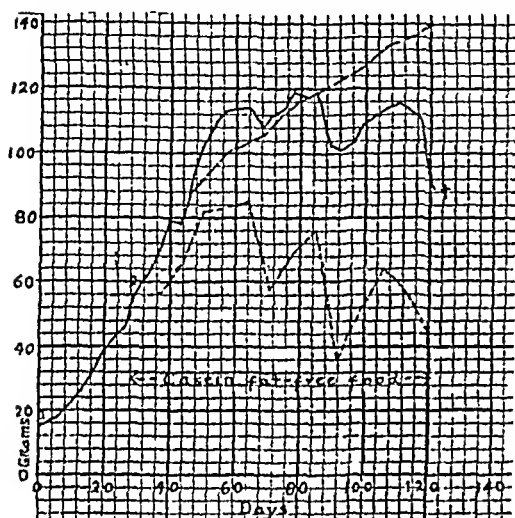


CHART 3 Rat 533, ♀, CHART 4, Rat 661, ♂ The food during the casein-fat-free period had the following percentage composition

Casein	22.0
Sucrose	20.0
Starch	28.5
Artificial protein free milk <sup>13</sup>	29.5
	<hr/>
	100.0

Illustrative charts of our feeding trials are introduced here. The ordinates of the curves represent body-weight (solid line) or food

<sup>13</sup> The successful use of this purely artificial product consisting of Ca, 1.97, Mg, 0.23, Na, 2.03, K, 2.66, PO<sub>4</sub>, 3.33, Cl, 4.13, SO<sub>4</sub>, 0.30, Fe, 0.04, Citric acid, 3.33, Lactose, 82.0 per cent, has been described by Osborne and Mendel, *Proc Soc of Exp Biol and Med*, 15, p 73, 1912. The relatively early failure to continue to grow, shown by chart 3, was caused by diseased lungs which terminated the life of Rat 533.

intake (dotted line) in grams, the abscissae represent days. The average (normal) curve of growth, plotted from body-weight data available for normally growing animals of the same sex, is represented by a broken line for comparison. In period 1 of all curves the rats were fed on ordinary mixed diet or by the mother

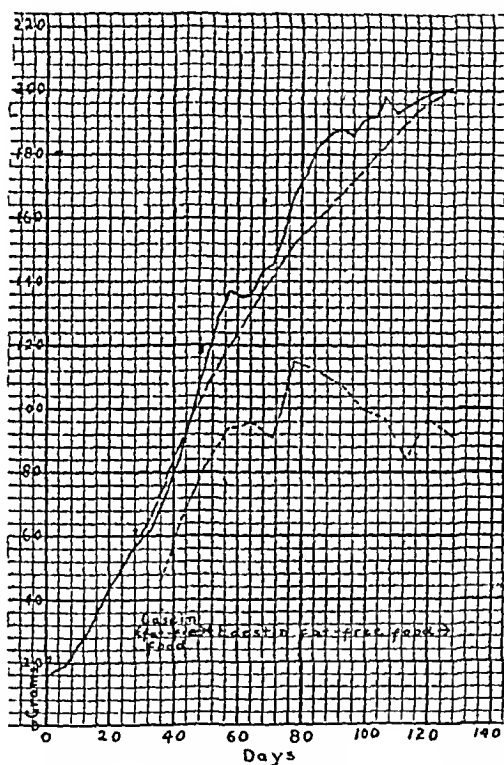


CHART 5, Rat 529, ♂ The fat-free diet had the following percentage composition

	Period 2	Period 3
Casein	22.0	0.0
Edestin	0.0	22.0
Sucrose	20.0	20.0
Starch	28.5	28.5
Artificial protein free milk	29.5	29.5
	100.0	100.0

In so far as one can judge by appearance and body weight these experiments with fat-free diets show growth quite as successful as



that attained with natural or artificial mixtures of all the types of food stuffs. Although we cannot claim a *complete* freedom from "lipoids" for the foods prepared as described above, it is scarcely

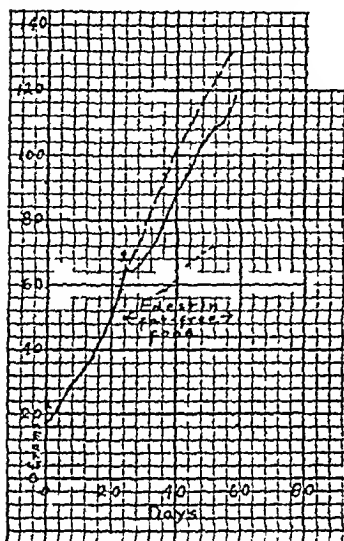


CHART 6, Rat 640, ♂ The fat-free food had the following percentage composition

Edestin	22 0
Sucrose	20 0
Starch	28 5
Artificial protein free-milk	29 5
	<hr/> 100 0

likely that products so carefully isolated can include any significant quantities of cerebrosides or phosphatides. This is peculiarly true of experiment 6 in which the sole possibilities of contamination are associated with the recrystallized phosphorus-free protein edestin and refined starch.

McCollum<sup>14</sup> has demonstrated that the phosphorus needed by an animal for phosphatide formation can be drawn from inorganic phosphates, and that phosphatides can be synthesized anew in the animal body. Rohmann<sup>15</sup> asserts the possibility of lecithin synthesis in mice which were maintained into the second generation on lecithin-free food. Our own experiments point in the same direction with regard to the lipoids in general, and they give positive evidence of the dispensableness of true fats for growth.<sup>16</sup>

<sup>14</sup> McCollum *Amer Journ of Physiol*, xxv, p 120, 1909, McCollum and Halpin *This Journal*, xi, 1912, *Proc Soc Biol Chem*, p xii, also Fingerling *Biochem Zeitschr*, xxxviii, p 438, 1912

<sup>15</sup> Röhmann *Biochemie*, 1908, p 109

<sup>16</sup> In agreement with Stepp, we have not yet succeeded similarly in inducing adequate growth in mice with similar diets. Stepp, who used crude food substances, is, however, cautious in his statements. He says "Wenn nach den mitgeteilten Versuchen und den anschliessenden Erörterungen der Schluss sich aufdrängt, dass gewisse alkohol-ätherlösliche Substanzen für die Ernährung von Mäusen unentbehrlich sind, so möchte ich diesen Schluss nicht ohne eine Einschränkung aufrechterhalten. Die Untersucher, die sich mit dem

The possibilities of the method of study introduced by us are manifest. The problems of the origin of fats in animals and their genesis from various carbohydrates or proteins are thus made approachable by experiment.<sup>17</sup> We hope to return to these questions later.

Studium der Lipoidbeschäftigten, haben, wie schon kurz erwähnt, an diesen Körpern Eigenschaften gefunden, die man in der Chemie bisher kaum kannte. Die Lipoiden haben eine ganz ausserordentliche Fähigkeit, auf die Löslichkeit anderer Stoffe einzuwirken und ihnen Löslichkeit in den spezifischen Lipoidlösungsmitteln zu verleihen, in denen die Stoffe sonst ganzlich unlöslich sind. So wäre es nicht undenkbar, dass gemeinschaftlich mit den Lipoiden irgendwelche unbekannte lebenswichtige Stoffe in Lösung gehen und dass so die Lipoiden gewissermassen zu Trägern für diese Stoffe wurden, dass mit anderen Worten bei der Entfernung von Lipoiden die unbekannten Körper mit entfernt und bei Zusatz von Lipoiden mit diesen zugesetzt werden. Ein Hinweis auf eine derartige Möglichkeit erscheint notwendig, solange es nicht gelingt, die Versuche mit chemisch reinen Körpern durchzuführen."

<sup>1</sup> Lummert (*Pflüger's Archiv*, LV, p. 176, 1898) has made attempts in the same direction.



## RESEARCHES ON PURINES

### ON 2-OXY-6,8,9-TRIMETHYLPURINE, 2-OXY-6,9-DIMETHYLPURINE AND 2-OXY-8,9-DIMETHYLPURINE

(SEVENTH PAPER)<sup>1</sup>

By CARL O. JOHNS

(From the Sheffield Laboratory of Yale University)

(Received for publication, May 21, 1912)

It is rather remarkable that not one of the many possible isomerides of the monoxy-trimethyl-purines has hitherto been described. If compounds of this type are produced by hydrolytic or enzymotic action on nucleoproteins they might easily be overlooked, since a purine derivative containing three methyl groups and only one atom of oxygen would probably be very soluble in water and consequently would be very difficult to isolate. 2-Oxy-6,8,9-trimethylpurine, which is described in this communication, is rather soluble in water notwithstanding the fact that two of the three methyl groups are attached to carbon atoms. If two of the methyl groups were attached to nitrogen atoms in the purine molecule the solubility would undoubtedly be increased to a high degree.

When 2-oxy-4-methyl-5-amino-6-methyl-aminopyrimidine (I)<sup>2</sup> was heated with acetic anhydride the result was a quantitative yield of the corresponding monoacetyl-pyrimidine. When this compound was heated at 225° to 230°C water was evolved and 2-oxy-6,8,9-trimethylpurine (II) was produced. This purine contained two molecules of water and gave a picrate that decomposed at 253°C.

Only one of the thirteen isomerides of 2-oxy-dimethylpurine has been described, namely, 2-oxy-3,7-dimethylpurine (IV) which was synthesized by Tafel<sup>3</sup>. This paper contains a description of two new 2-oxy-dimethylpurines which were obtained as follows

<sup>1</sup> This *Journal*, xi, p 393, 1912

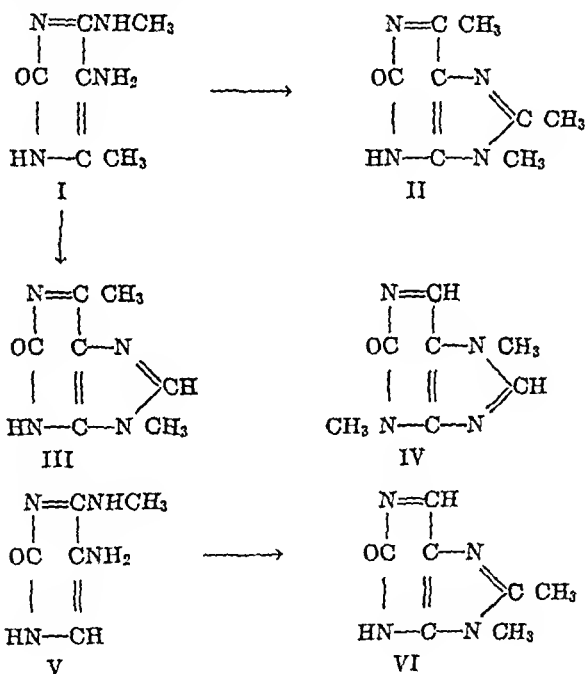
<sup>2</sup> Johns This *Journal*, xi, p 397, 1912

<sup>3</sup> Julius Tafel *Ber d deutsch chem Gesellsch*, xxxii, p 3201, 1899

2-Oxy-6,9-dimethylpurine (III) was prepared by the action of 85 per cent formic acid on 2-oxy-4-methyl-5-amino-6-methylaminopyrimidine (I). The yield was almost quantitative. The picrate decomposed with effervescence at 224°C.

When 2-oxy-5-amino-6-methylaminopyrimidine (V)<sup>4</sup> was heated with acetic anhydride at 150° to 160°C, a 90 per cent yield of 2-oxy-8,9-dimethylpurine resulted. This purine was easily soluble in cold water. It formed a picrate that decomposed at 233°C.

These researches will be continued.



#### EXPERIMENTAL PART

*Acetyl-2-oxy-4-methyl-5-amino-6-methylaminopyrimidine*,  $\text{C}_8\text{H}_{12}\text{O}_2\text{N}_4$ . Four grams of 2-oxy-4-methyl-5-amino-6-methylaminopyrimidine<sup>5</sup> were mixed with 40 cc of acetic anhydride and

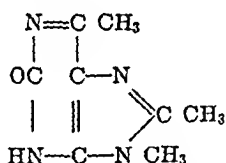
<sup>4</sup> Johns This Journal, ix, p 165, 1911

<sup>5</sup> Johns Loc cit

the mixture was evaporated to dryness on a steam bath. At first a bulky compound was formed but this gradually changed to a granular substance as the evaporation proceeded. At no time did complete solution take place. When the evaporation had become complete a little alcohol was added and the evaporation was repeated in order to remove the last traces of acetic anhydride. The residue was readily soluble in cold water or hot alcohol and from the latter solvent it crystallized out almost completely on cooling, forming a bulky mass of hair-like crystals. These began to shrink at about 250°C and turned dark slowly above that temperature and finally decomposed rapidly at 290° to 300°C. The yield was almost quantitative.

N	Calculated for	Found
	$C_8H_{12}O_2N_4$	
	28.57	28.83

*2-Oxy-6,8,9-trimethylpurine*



Four grams of acetyl-2-oxy-4-methyl-5-amino-6-methylamino-pyrimidine were pulverized and heated in an oil bath at 225° to 230°C until water ceased to escape. The resulting residue was dissolved in hot water and the solution was decolorized with blood coal. On cooling this solution, colorless, hair-like crystals were obtained. These crystals were easily soluble in hot and moderately soluble in cold water. They were easily soluble in hot and sparingly soluble in cold alcohol and almost insoluble in boiling benzene. They decomposed at about 275°C. The yield was 80 to 90 per cent of the calculated. The crystals obtained from the aqueous solution formed a mat and were difficult to dry at room temperature and when dried for several days in a desiccator over sulphuric acid they still retained two molecules of water of crystallization.

- I 1.0787 gram of substance lost 0.1877 gram at 130°C  
 II 1.8153 gram of substance lost 0.3211 gram at 130°C

	Calculated for $C_8H_{10}ON_4 \cdot 2H_2O$	Found	
$H_2O$	16 82	I 17 40	II 17 60
0 2064 gram of anhydrous substance gave 0 4086 gram of $CO_2$ and 0 1045 gram of $H_2O$			

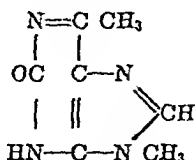
	Calculated for $C_8H_{10}ON_4$	Found
C	53 93	53 99
H	5 61	5 62
N	31 46	31 45

An aqueous solution of the purine did not give precipitates with barium chloride or platonic chloride

The picrate,  $C_8H_{10}ON_4 \cdot C_6H_2(NO_2)_3OH$  This salt crystallized out slowly when a solution of picric acid was added to an aqueous solution of 2-oxy-6,8,9-trimethylpurine The crystals were columnar in shape and were moderately soluble in hot and difficultly soluble in cold water They began to darken at about  $240^\circ C$  and effervesced violently at  $253^\circ C$

	Calculated for $C_8H_{10}ON_4 \cdot C_6H_2(NO_2)_3OH$	Found
N	24 08	24 17

*2-Oxy-6,9-dimethylpurine*



Two grams of 2-oxy-4-methyl-5-amino-6-methylaminopyrimidine were dissolved in 20 cc of 85 per cent formic acid and the solution was evaporated to dryness on a steam bath The residue was dissolved in dilute ammonia and the solution was decolorized with blood coal After boiling off most of the ammonia, the solution was acidified with acetic acid On cooling, a bulky precipitate composed of anhydrous, hair-like crystals was obtained These dissolved in about 400 parts of boiling water and dissolved but slightly in cold water or boiling alcohol They were not soluble in boiling benzene They did not melt at  $320^\circ C$  The yield was almost quantitative An aqueous solution failed to give precipitates with barium chloride or platonic chloride

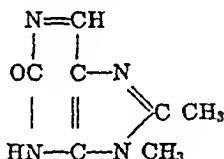
0.2001 gram of substance gave 0.3785 gram of  $\text{CO}_2$  and 0.0865 gram of  $\text{H}_2\text{O}$

	Calculated for $\text{C}_7\text{H}_8\text{ON}_4$	Found	
C	51.22	51	58
H	4.87	4	80
N	34.14	<sup>I</sup> 34.08	<sup>II</sup> 34.19

The picrate,  $\text{C}_7\text{H}_8\text{ON}_4 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$  This salt was prepared by adding a cold, saturated, aqueous solution of picric acid to a hot, saturated solution of 2-oxy-6,9-dimethylpurine. On cooling, the picrate deposited as long, slender prisms that decomposed with effervescence at  $224^\circ\text{C}$ .

	Calculated for $\text{C}_7\text{H}_8\text{ON}_4 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$	Found
N	24.93	24.91

*2-Oxy-8,9-dimethylpurine*



Four grams of pulverized 2-oxy-5-amino-6-methylaminopyrimidine<sup>6</sup> were heated with 25 cc of acetic anhydride for one hour in an oil bath which was kept at  $150^\circ$  to  $160^\circ\text{C}$ . The resulting solution was evaporated to dryness on a steam bath and the residue was heated for an hour at  $130^\circ$  to  $140^\circ\text{C}$ . After dissolving in hot water and clarifying, on cooling rapidly a bulky mass of slender prisms was obtained. When the solution was cooled slowly the purine crystallized in beautiful, rather stout prisms. These were very soluble in hot and moderately soluble in cold water. They were difficultly soluble in hot alcohol and insoluble in boiling benzene. They did not melt at  $320^\circ\text{C}$ . The yield was 80 to 90 per cent of the calculated. A cold concentrated solution did not give a precipitate with barium chloride but gave a difficultly soluble precipitate with platonic chloride. The free base probably contained water of crystallization but this escaped slowly on drying.

<sup>6</sup> Johns *Loc cit*



over sulphuric acid or by exposing the crystals to the air for a long time, hence, attempts to determine water gave discordant results. The specimen used for the following analyses was dried at 130° to 140°C

0.1656 gram of substance gave 0.3091 gram of  $\text{CO}_2$  and 0.0709 gram of  $\text{H}_2\text{O}$

	Calculated for $\text{C}_7\text{H}_8\text{ON}_4$	Found	
C	51.22	50.90	
H	4.87	4.75	
N	34.14	I 34.10	II 34.13

*The picrate*,  $\text{C}_7\text{H}_8\text{ON}_4 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$ . This compound precipitated immediately as a bulky mass of needles on adding a solution of picric acid to a cold, saturated solution of 2-oxy-8,9-dimethylpurine. When the picrate was recrystallized from water it formed small, stout blocks. These decomposed with effervescence at 233°C.

	Calculated for $\text{C}_7\text{H}_8\text{ON}_4 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$	Found
N	24.93	24.70

# PHYTIN AND PYROPHOSPHORIC ACID ESTERS OF INOSITE

## SECOND PAPER

By R. J. ANDERSON

(From the Chemical Laboratory of New York Agricultural Experiment Station, Geneva, N. Y.)

(Received for publication, May 22, 1912)

In the last report<sup>1</sup> from this laboratory on the subject of phytin, various salts of phytic acid were described, as well as the tetraphosphoric acid ester of inosite. Since then the investigation has been continued in connection with another problem dealing with the form in which phytin exists in wheat bran, which is not yet finished, but as the present work is closely related to that reported earlier, it seems advisable to publish it at this time.

In addition to the salts of phytic acid described before, the following have been prepared:

The calcium-magnesium-potassium phytate,  $C_6H_{12}O_{27}P_6Ca_3Mg_2K_2$ , a white amorphous powder obtained by neutralizing a solution of calcium-magnesium phytate in dilute hydrochloric acid with potassium hydroxide.

The penta-calcium phytate,  $C_6H_{14}O_{27}P_6Ca_5$ , is obtained as a white powder on precipitating an aqueous solution of phytic acid with calcium acetate.

The tetra-calcium phytate,  $C_6H_{16}O_{27}P_6Ca_4 + 12H_2O$ , is obtained as a white semicrystalline or fine granular powder when the above penta-calcium phytate in dilute hydrochloric acid solution is evaporated in vacuum in the presence of calcium acetate.

The penta-magnesium phytate,  $C_6H_{14}O_{27}P_6Mg_5 + 24H_2O$ , is obtained as a crystalline powder when an aqueous solution of phytic acid and excess of magnesium acetate is evaporated in vacuum.

<sup>1</sup> This *Journal*, **41**, p. 471, 1912, and Technical Bulletin 19 of the N. Y. Agricultural Experiment Station.

A copper salt,  $C_6H_{12}O_{27}P_6Cu_6$ , corresponding to a hexa-cupric phytate is obtained when phytic acid is precipitated with copper acetate

The octa-silver phytate,  $C_6H_{16}O_{27}P_6Ag_8$ , is precipitated as a white amorphous powder by alcohol from an aqueous solution of phytic acid containing twelve equivalents of silver nitrate

The hepta-silver phytate,  $C_6H_{17}O_{27}P_6Ag_7$ , results when the dilute nitric acid solution of the above octa-silver phytate is precipitated with alcohol

Since various attempts to synthesize phytic acid or to prepare a hexa-phosphoric acid ester of inosite by acting on inosite with phosphoric acid lead only to the formation of the tetra-phosphoric acid ester of inosite,<sup>2</sup> it seemed of interest to determine what products would be formed when acting on inosite with pyrophosphoric acid. If phytin were a complex pyrophosphoric acid compound of inosite as suggested by Starkenstein<sup>3</sup> it appeared not impossible to synthesize it from these constituents. Such a synthesis would be of considerable theoretical and scientific value in connection with the chemistry of phytin and would also furnish an additional proof of the presence of pyrophosphoric acid compounds in nature

Several futile efforts were made in this direction but it was found that the reactions tried lead only to pyrophosphoric acid esters of inosite. These esters are very easily formed but their purification is very difficult

When acting on dry inosite (1 molecule) with dry pyrophosphoric acid (3 molecules or sufficient to form phytic acid) at a temperature of  $200^{\circ}$ – $220^{\circ}$  C a new and stable ester is formed. On analysis, results were obtained corresponding to a di-pyrophosphoric acid ester of inosite, a compound isomeric with the tetra-phosphoric acid ester described in a former paper

Attempts to isolate the reaction product by the method described for the tetra-phosphoric ester,<sup>4</sup> that is, by precipitating as a barium salt with alcohol in the presence of hydrochloric acid, failed at first because barium pyrophosphate is as insoluble in acidified dilute alcohol as is barium phytate, for instance, or as are the pyrophos-

<sup>2</sup> Anderson *loc cit*

<sup>3</sup> *Biochem Zeitschr*, xxv, p 56

<sup>4</sup> Anderson *loc cit*

phoric acid esters Various other salts were tried with negative results, the pyrophosphate invariably was precipitated at the same time

As is well known, pyrophosphoric acid when boiled with dilute mineral acids is very easily transformed into orthophosphoric acid The isolation of the new ester was made possible by taking advantage of this property

In the last paper<sup>5</sup> it was reported that phytin, when dry and free from mineral acids, is stable, that drying at 115°C caused no appreciable decomposition and that no inosite could be isolated from 100 grams of phytin after drying to constant weight at this temperature

Experience since then has shown that phytin may be boiled for hours in dilute hydrochloric or sulphuric acid without suffering marked decomposition In fact it may be boiled for days with 30 per cent sulphuric acid without a determinable quantity of inosite being formed This seemed strange as various other investigators have emphasized the fact that phytin is very easily hydrolyzed and that even in water it suffers a more or less rapid decomposition

The action of nitric acid seems to cause a more rapid decomposition for even the purest phytin when warmed in dilute nitric acid solution with ammonium molybdate gives very quickly the characteristic yellow precipitate of ammonium-phosphomolybdate Several days, however, are required to cause complete decomposition in dilute nitric acid solution at a temperature of 60°-70°C Quantitative experiments to measure the rate of decomposition have not been carried out, but they could be very easily as the change is very slow

The following will illustrate this point

In an analysis of two different phytin preparations the substance was boiled with concentrated nitric acid under occasional additions of concentrated hydrochloric acid for about half an hour The organic matter was destroyed apparently at the end of this time as the solution was practically colorless The phosphorus was determined in this solution by the usual molybdate method After keeping at a temperature of 60°C for one hour the precipitate was filtered off and the filtrate again warmed on the water-bath for another hour A new portion of the yellow precipitate had then formed which was removed by filtration and the filtrate again warmed on

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<sup>5</sup> Anderson *loc cit*

the water-bath. A yellow precipitate continued to form slowly but continuously for two days when the experiment was discontinued. During this time the water lost by evaporation was replaced from time to time and small quantities of nitric acid were also added. The phosphorus when determined in the first precipitate and in that which formed during the first day amounted to 9.92 and 10.25 per cent only, whereas when determined after first destroying the organic matter by the Neumann method 14.42 and 15.23 per cent respectively were found.

In another case 100 grams of calcium phytate were boiled under a reflux condenser with about 300 cc. of 30 per cent sulphuric acid continuously for one day, over night it was heated on the water-bath and the next day the boiling was continued all day. After precipitating with excess of barium hydroxide, thorough washing in hot water, removal of excess of barium by carbon dioxide and evaporating on the water-bath, no inosite could be found in the slight residue which remained.

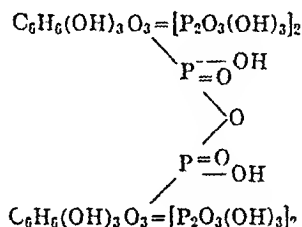
To determine if the phytin molecule suffered any partial decomposition on boiling with dilute acids 1 gram of phytic acid, dissolved in 100 cc. of water acidified with 10 cc. of 5 N hydrochloric acid, was boiled over a free flame for one hour. After cooling, barium chloride was added and the barium phytate precipitated by the addition of alcohol. The substance was twice purified by precipitating its hydrochloric acid solution with alcohol. On analysis, results were obtained which showed that the substance was a pure tri-barium phytate, the salt which is always obtained under the above conditions of precipitation.

In view of this relative stability of the phytin molecule it was thought that the pyrophosphoric acid ester referred to above might be more stable than the pyrophosphoric acid in the reaction mixture. Qualitative experiments showed that this was actually the case.

An aqueous solution of pyrophosphoric acid, acidified with hydrochloric acid and a solution of the above inosite-pyrophosphoric acid reaction-mixture, also acidified with hydrochloric acid, were boiled for one hour. Some barium chloride and a like volume of alcohol were then added. The solution containing only pyrophosphoric acid gave no precipitate, while before boiling, alcohol produced at once a white precipitate of barium pyrophosphate. The solution containing the inosite-pyrophosphoric acid reaction-mixture gave a white flocculent precipitate—the barium salt of the new ester.

As the excess of the pyrophosphoric acid was present as orthophosphoric acid after boiling, it did not interfere with the purification of the compound by the dilute acid alcohol method.

By acting on dry inosite (1 molecule) with dry pyrophosphoric acid (6 molecules) at a temperature of 200°-220°C another new pyrophosphoric ester was obtained. After boiling, as before, with dilute hydrochloric acid and purifying as the barium salt this product was found to be a di-inosite tri-pyrophosphoric acid ester, that is, its molecule is evidently made up of 2 molecules of di-pyrophosphoric acid esters of inosite joined through 1 molecule of pyrophosphoric acid and, accordingly, it corresponds with the following formula



It is evident therefore that complex compounds such as phytic acid is supposed to be cannot be formed at elevated temperatures, as in the various reactions tried in these experiments only esters were produced, and at lower temperatures apparently no reaction takes place. These compounds are in physical and chemical properties very similar to phytic acid. They form analogous acid salts which in appearance and solubility seem almost identical with salts of phytic acid. Whether esters, such as above, are found in nature is at present unknown. It is, however, not impossible that a part of the organically bound phosphorus existing in plants may be present in some such, or similar, forms.

The silver salts previously referred to were prepared in the hope that they might serve for the preparation of an ester of phytic acid with which molecular weight determinations might be made. As was to be expected, however, only acid salts were obtained and, as such, were quite useless for the purpose in view. In the reaction between phytic acid and silver nitrate, nitric acid is of course liberated and when any strong acid is present only acid phytates are obtained.

Efforts made to prepare an ester by acting on sodium phytate with methyl-sulphate proved useless as ~~no ester~~ <sup>no ester</sup> could be isolated.

Further experiments along this line are contemplated and will be reported later

In an article concerning the phosphorus compounds found in food materials which appeared in a Swedish chemical journal little known in this country and which is not abstracted by any of the larger chemical journals, a valuable contribution to the chemistry of phytin was made by A Rising<sup>6</sup> Among other things he describes a silver phytate of the following composition C, 55, H, 108, P, 132 and Ag 5265 per cent, from which results he concludes that it must represent a complex pyrophosphoric acid compound of inosite It is noteworthy that this author and E Starkenstein,<sup>7</sup> independently and practically at the same time, expressed the same opinion, viz that phytin represents a complex pyrophosphoric acid compound of inosite

The silver salt described by Rising corresponds to the hepta-silver phytate mentioned in this paper He proposes the empirical formula,  $C_6H_{14}Ag_6P_5O_{22}$ , but his results agree equally well with a hepta-silver phytate,  $C_6H_{17}O_{27}P_6Ag_7$

	CALCULATED	FOUND BY RISING	FOUND FOR HEPTA-SILVER PHYTATE IN THIS LABORATORY
C	4 92	5 50	
H	1 16	1 08	
P	12 72	13 20	13 02
Ag	51 64	52 65	52 43

From the above there appears to be no doubt that these salts are identical

The several salts of phytic acid reported in this paper were prepared from previously purified and analyzed phytic acid and for this reason it was deemed quite superfluous to make carbon and hydrogen determinations on each salt

<sup>6</sup> *Svensk Kemisk Tidskrift*, xxii, 7, p 143, 1910 I am indebted to Mr A R Rose of Columbia University for this as well as for many other valuable references

<sup>7</sup> *Loc cit*

## EXPERIMENTAL PART

*Calcium magnesium potassium phytate*

Two grams of phytic acid were dissolved in about 100 cc of water, 0.224 gram of MgO (2 molecules), and 0.84 gram CaCO<sub>3</sub> (3 molecules) added. The MgO dissolved at once and nearly all the calcium carbonate but the salt of phytic acid was precipitated at the same time as a white precipitate. This was dissolved by a few drops of hydrochloric acid, the solution filtered and the filtrate rendered slightly alkaline to litmus with potassium hydroxide. After the precipitate had settled it was filtered off, washed well in 50 per cent alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a fine white amorphous powder. It was free from chlorine. On moist litmus paper it showed a faintly alkaline reaction. It was slightly soluble in water but readily soluble in dilute acids. Yield, 2.9 grams.

After drying at 105° in vacuum over phosphorus pentoxide it was analyzed

	For C <sub>6</sub> H <sub>12</sub> O <sub>17</sub> P <sub>6</sub> Ca <sub>3</sub> Mg <sub>2</sub> K <sub>2</sub> = 948
Calculated	Ca, 12.65, Mg, 5.12, K, 8.24, P, 19.60 per cent
Found	Ca, 13.03, Mg, 4.29, K, 6.42, P, 19.07 per cent

This shows the difficulty of obtaining pure salts of phytic acid when several bases are combined in the same molecule of the salt.

*Penta-calcium phytate*

One gram of phytic acid was dissolved in about 50 cc of water and excess of calcium acetate added. On the first addition of the calcium acetate a white precipitate is produced, but on shaking, this redissolves and only after a liberal excess of the acetate has been added is the precipitate permanent. After settling, the product was filtered and thoroughly washed in 50 per cent alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The substance was a perfectly white amorphous powder. On moist litmus paper it showed an acid reaction. It is only slightly soluble in water, readily soluble in dilute mineral acids, less soluble in acetic acid.



For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide

	For $C_8H_{14}O_{17}P_6Ca_3 = 904$	
Calculated		Ca, 22 12, P, 20 57 per cent
Found		Ca, 22 46, P, 20 62 per cent

### *Tetra-calcium phytate*

Various attempts were made to obtain a penta-calcium phytate in crystalline form without success. A tetra-calcium phytate was finally obtained by the following method.

The penta-calcium phytate was dissolved in a small quantity of 0.5 per cent hydrochloric acid, a concentrated solution of calcium acetate was added until a permanent precipitate remained which was then dissolved by the addition of a few drops of dilute hydrochloric acid. On now concentrating in vacuum to somewhat less than half the bulk at a temperature of  $40^{\circ}$  the calcium salt separates. The product was filtered off, washed thoroughly in 50 per cent alcohol and ether and dried in the air. The substance was a white semicrystalline or fine granular powder of irregular form. Its solubility was practically the same as for the penta-calcium phytate. It was free from chlorine.

On drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide the substance lost water corresponding to  $12 H_2O$ .

0.2120 gram substance	0.0422 gram $H_2O$
0.1238 gram substance	gave 0.0308 gram $CaO$ and 0.0967 gram $Mg_2P_2O_7$
0.1857 gram substance	gave 0.0456 gram $CaO$ and 0.1448 gram $Mg_2P_2O_7$
	For $C_8H_{16}O_{17}P_6Ca_4 = 866$

Calculated	Ca, 18 47, P, 21 47 per cent
Found	{ Ca, 17 78, P, 21 77 per cent
	{ Ca, 17 55, P, 21 73 per cent

For  $12 H_2O$ , calculated 19.96, found 19.90 per cent

### *Penta-magnesium phytate*

Two and five-tenths grams phytic acid were dissolved in about 100 cc. of water and a concentrated solution of magnesium acetate was added. This did not cause any precipitate nor could the substance be brought to crystallization by any of the usual methods. The solution was then concentrated to about half its bulk in vacuum.

at a temperature of  $35^{\circ}$  to  $40^{\circ}$ . As the concentration proceeded the substance began to separate as a heavy powder. This was filtered off, well washed in dilute alcohol, alcohol and ether and dried in the air. The product was a perfectly white semi-crystalline or loose granular powder of irregular form. On moist litmus paper it showed an acid reaction. It was slightly soluble in water, readily soluble in acids.

For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide. It lost water corresponding to  $24 \text{ H}_2\text{O}$ .

0.1504 gram substance gave 0.0510 gram  $\text{H}_2\text{O}$

0.0997 gram substance gave 0.0671 gram  $\text{Mg}_2\text{P}_2\text{O}_7$  for Mg

0.0498 gram substance gave 0.0393 gram  $\text{Mg}_2\text{P}_2\text{O}_7$  for P

For  $\text{C}_6\text{H}_{14}\text{O}_{17}\text{P}_2\text{Mg}_3 = 825.5$

Calculated Mg, 14.71, P, 22.53 per cent

Found Mg, 14.69, P, 21.99 per cent

For  $24 \text{ H}_2\text{O}$ , calculated 34.36, found 33.91 per cent

### *Hexa-copper phylate*

This salt is precipitated directly from phytic acid solutions by copper acetate. It is difficult, however, to obtain a pure compound as it is apt to contain either too little or too much copper, depending upon the conditions under which the precipitate is formed. In the purification of phytic acid it is usual to remove other bases which are present by repeatedly precipitating with barium chloride, the barium salt which is finally obtained is then decomposed with sulphuric acid. It is found, however, that if only the calculated quantity of sulphuric acid is used the barium sulphate which is formed is in such an extremely fine condition that it is impossible to remove it completely either by repeated filtrations or even by long centrifuging. But if a slight excess of sulphuric acid is used the barium sulphate in the course of only a few hours becomes heavy and granular and may be easily removed by simple filtration. In order to get rid of the excess of sulphuric acid the solution is now precipitated with copper acetate. The copper salt can be easily washed free of the sulphate and acetate with water as it is very slightly soluble in very dilute acids. The pure copper salt is then easily decomposed with hydrogen sulphide and the free phytic acid obtained.

The copper phytate obtained from such slightly acid solutions was analyzed and the following results obtained

	For $C_6H_1 O_{27}P_6Cu_6 = 1083$
Calculated	Cu, 35 18, P, 17 17 per cent
Found	Cu, 33 54, P, 16 88 per cent

Pure phytic acid in water was precipitated with pure copper acetate when a compound was obtained which had the following composition

Cu, 37 57, P, 15 13 per cent

It is seen from above that from slightly acid solutions of phytic acid a copper salt is precipitated which contains somewhat too little copper while from an aqueous phytic acid solution a salt is obtained which contains over 2 per cent excess of copper

The copper phytate is, like all other phytates, exceedingly soluble in 10 per cent phytic acid. It dissolves readily until a thick heavy syrup is formed, but it was found impossible to bring this solution to crystallization. Both of the above copper salts show an acid reaction on moist litmus paper. The red color is only developed slowly and is probably due to hydrolysis

#### *Octa-silver phytate*

This salt is obtained when an aqueous solution of phytic acid, mixed with twelve equivalents of silver nitrate, is precipitated with alcohol. The product is a heavy, white flocculent precipitate which settles at once. It was filtered off, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product is only slightly affected by light but on continued exposure turns first yellowish and later dark in color. In the dry state it is a heavy white amorphous powder of acid reaction on moist litmus paper. It is very soluble in dilute nitric acid and exceedingly soluble in phytic acid. Many attempts were made to bring it to crystallization from the latter solution without success.

For analysis it was dried at  $105^\circ$  in vacuum over phosphorus pentoxide

	For $C_6H_{16}O_{27}P_6Ag_8 = 1569$
Calculated	Ag, 55 00, P, 11 85 per cent
Found	Ag, 55 98 P 11 94 per cent

*Hepta-silver phytate*

This salt is obtained when the octa-silver phytate, dissolved in dilute nitric acid, is precipitated with alcohol. The precipitate after filtering, washing and drying as before was analyzed. In appearance and properties it was identical with the octa salt.

For  $C_8H_{17}O_7P_3Ag_7 = 1462$

Calculated

Ag, 51.64, P, 12.72 per cent

Found

Ag, 52.43, P, 13.02 per cent

*Di-pyrophosphoric acid ester of inositol*

Dry pyrophosphoric acid, 17.02 grams (little over 9 molecules), was heated in a flask in an oil bath to about  $200^\circ$  and 5.4 grams (3 molecules) of dry inositol added. At this temperature the inositol dissolved at once, forming a thick reddish-brown colored solution. After heating to  $220^\circ$  for a few minutes the flask was removed and allowed to cool. The reaction mixture was dissolved in 500 cc of water, 20 cc of 5 N hydrochloric acid added and the whole boiled for about one hour. At the end of this time the excess of the pyrophosphoric acid has become changed to ortho-phosphoric acid and as such does not interfere with the precipitation of the barium salt of the ester with alcohol.

After cooling the above solution containing the new ester it was diluted to 1 liter with water, a solution of 40 grams of barium chloride in water was added and the barium salt of the ester was then precipitated by adding 1 liter of alcohol. The resulting precipitate was filtered off at once and for the purpose of removing adhering inorganic phosphate was precipitated twice from 0.5 per cent hydrochloric acid, in the presence of a small quantity of barium chloride, with alcohol and then twice from the same strength hydrochloric acid with alcohol. After finally filtering and thoroughly washing in 50 per cent alcohol, alcohol and ether it was dried in vacuum over sulphuric acid. The product so obtained was a white amorphous powder. In appearance it was very similar to the tribarium phytate and the barium salt of the tetra-phosphoric acid ester of inositol except that when precipitated with alcohol the particles appeared coarser. On moist litmus paper it showed strong acid reaction. It was readily soluble in dilute hydrochloric and nitric acids, less soluble in acetic acid, very slightly soluble in water and exceedingly

soluble in 10 per cent phytic acid It was free from chlorine  
Yield, 11.8 grams

After drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide the substance was analyzed

0.2617 gram substance gave 0.0421 gram  $H_2O$  and 0.1016 gram  $CO_2$   
 0.2796 gram substance gave 0.0488 gram  $H_2O$  and 0.1080 gram  $CO_2$   
 0.2566 gram substance gave 0.1495 gram  $BaSO_4$  and 0.1443 gram  $Mg_2P_2O_7$   
 Found  $\left\{ \begin{array}{l} C, 10.58, H, 1.80, P, 15.67, Ba, 34.28 \text{ per cent} \\ C, 10.53, H, 1.95 \text{ per cent} \end{array} \right.$

The substance was not yet pure being probably mixed with some mono-pyrophosphoric acid ester of inosite, at least the high carbon and low phosphorus points to such a conclusion

It was hoped that the exceeding solubility of the substance in phytic acid might serve to separate these bodies For this purpose the whole substance was dissolved in 20 cc of 10 per cent phytic acid On diluting with water a portion of the substance separated as a heavy granular powder To complete the separation 100 cc of water was added and then allowed to stand two days at room temperature This precipitate was discarded, as analysis after purifying by precipitating from 0.5 per cent hydrochloric acid with alcohol, showed that it was still impure and only 0.9 gram had been obtained The great bulk of the substance was accordingly contained in the filtrate from the above This filtrate was diluted to 300 cc with water and then precipitated by adding 300 cc of alcohol The voluminous white precipitate was filtered off, washed thoroughly in 50 per cent alcohol and alcohol For purification it was dissolved in 0.5 per cent hydrochloric acid and precipitated by alcohol After filtering and thoroughly washing in dilute alcohol until free from chlorine it was washed in alcohol and ether and dried in vacuum over sulphuric acid The product so obtained was a pure white amorphous powder On moist litmus paper it showed a strong acid reaction The solubility corresponded with that previously observed Yield, 7.7 grams

For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide

0.2914 gram substance gave 0.0440 gram  $H_2O$  and 0.1026 gram  $CO_2$   
 0.1667 gram substance gave 0.0977 gram  $BaSO_4$  and 0.0960 gram  $Mg_2P_2O_7$   
 For  $C_6H_6(OH)_2O_2(P_2O_5HBa)_2 = 770.7$   
 Calculated  $\left\{ \begin{array}{l} C, 9.34, H, 1.55, P, 16.08, Ba, 35.64 \text{ per cent} \\ C, 9.60, H, 1.68, P, 16.05, Ba, 34.48 \text{ per cent} \end{array} \right.$   
 Found

The barium was found to be a little low but this is compensated for through a slightly high carbon content, moreover it is sometimes difficult to obtain amorphous salts of this kind which show closer agreement than the above. The analysis leaves no doubt that the substance was the barium salt of the ester in question. It will be noticed that the di-pyrophosphoric acid ester is isomeric with the tetra-phosphoric acid ester of inosite previously referred to. Lack of time has prevented the determination of the free alcoholic (OH) groups in the inosite ring in either of these compounds. Experiments along this line are contemplated, however.

That the reaction between the inosite and the pyrophosphoric acid actually occurred along the lines discussed above may be judged by the amount of water given off. To determine this point 0.36 grams inosite (1 molecule) and 1.06 gram pyrophosphoric acid (3 molecules), both previously dried at  $100^{\circ}\text{C}$ , were heated in a small flask in an oil bath at  $200^{\circ}$ – $220^{\circ}$  under the same conditions as in the above experiment. The water, which began to come over at a temperature of about  $200^{\circ}$ , was collected in a weighed calcium-chloride tube. The aqueous vapors were removed by means of the suction pump but no special effort was made to secure quantitative results. The water obtained weighed 0.0494 gram whereas the quantity calculated for 2 molecules  $\text{H}_2\text{O}$  is 0.072 gram. The amount obtained is therefore only about 68 per cent of the theory.

#### *Preparation of the free di-pyrophosphoric ester*

The air-dried barium salt of the ester (4 gram) was suspended in water and decomposed with a slight excess of sulphuric acid, the barium sulphate was removed and the solution precipitated with copper acetate. The copper salt was filtered off, thoroughly washed in water, suspended in water and decomposed with hydrogen sulphide. It was found impossible to remove the copper sulphide completely by filtration as it formed a colloidal solution, but by acidifying with a few drops of hydrochloric acid and heating to boiling the copper sulphide was precipitated. After filtering, the filtrate was evaporated several times in vacuum for the removal of the hydrochloric acid and finally dried in vacuum over sulphuric acid and potassium hydroxide until it was of a thick syrupy consistency. The product obtained was of the same appearance as phytic

acid or the tetra-phosphoric ester, viz a thick light amber colored liquid After drying at  $105^{\circ}$  the substance was analyzed

0.1709 gram substance gave 0.0552 gram  $H_2O$  and 0.0892 gram  $CO_2$

0.1739 gram substance gave 0.1517 gram  $Mg_2P_2O_7$

For  $C_6H_8(OH)_4O_2(P_2O_5(OH))_2 = 500$

Calculated C, 14.40, H, 3.20, P, 24.80 per cent

Found C, 14.23, H, 3.61, P, 24.31 per cent

### *Properties of the free di-pyrophosphoric ester*

The concentrated aqueous solution is a thick light amber colored syrup On longer drying over sulphuric acid it becomes a hard and brittle hygroscopic mass

The aqueous solution is of strong acid reaction and sharp acid taste

With barium chloride no precipitate is produced either in the cold or on heating, alcohol or ammonia produces a white precipitate in this solution

Calcium chloride gives no precipitate even on heating but alcohol causes in this solution a voluminous flocculent precipitate

Calcium acetate produces at once a white precipitate sparingly soluble in acetic but readily soluble in mineral acids

Magnesium acetate gives a white precipitate readily soluble in acids

Ferric chloride gives a white or faintly yellowish precipitate very sparingly soluble in acids

Barium acetate gives a white precipitate sparingly soluble in acetic acid but readily soluble in dilute hydrochloric or nitric acids

Dilute silver nitrate does not cause a precipitate but concentrated silver nitrate gives a white precipitate

With ordinary molybdate solution no precipitate is produced but neutral molybdate gives a white precipitate which slowly turns yellowish in color On drying at  $105^{\circ}$  the substance turns very dark in color

### *Inosite from di-pyrophosphoric ester*

The free ester, 0.65 gram, was heated with 20 cc of 5N sulphuric acid in a sealed tube to  $150^{\circ}$  for about three hours The inosite

was isolated by the usual method and crystallized from dilute alcohol after addition of ether. After recrystallizing from hot dilute alcohol, adding ether and allowing to stand several hours in the cold, the substance was obtained in small, colorless crystals free from water of crystallization. Yield, 0.18 gram or 75 per cent of the theory. The air-dried, water-free substance melted at  $221^{\circ}\text{C}$  (uncorrected) and it gave the reaction of Scherer. Drying at  $110^{\circ}$  for one hour caused no loss of weight. On analysis the following results were obtained:

0.1634 gram substance gave 0.0981 H<sub>2</sub>O and 0.2374 gram CO<sub>2</sub>

For  $\text{C}_6\text{H}_{11}\text{O}_8 = 180$

Calculated

C, 40.00, H, 6.66 per cent

Found

C, 39.62, H, 6.71 per cent

#### *Di-inosite tri-pyrophosphoric acid ester*

This ester is formed when dry inosite is heated with excess of pyrophosphoric acid. The molecule of the new ester evidently consists of 2 molecules of di-pyrophosphoric acid esters of inosite joined by 1 molecule of pyrophosphoric acid.

Dry inosite, 1.8 grams (1 molecule), was heated with 10.7 grams (6 molecules) of pyrophosphoric acid under the same conditions as described for the di-pyrophosphoric ester and it was isolated as the barium salt in exactly the same way. After precipitating five times from 0.5 per cent hydrochloric acid with alcohol the product was finally obtained as a perfectly white amorphous powder. Its solubilities corresponded practically with those mentioned for the di-pyrophosphoric acid ester and likewise it showed a strong acid reaction on moist litmus paper.

For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.2086 gram substance gave 0.0296 gram H<sub>2</sub>O and 0.0614 gram CO

0.1412 gram substance gave 0.0879 gram BaSO<sub>4</sub> and 0.0844 gram Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>

Found

C, 8.02, H, 1.58, P, 16.66, Ba, 36.63 per cent

This substance was then again precipitated twice from 0.5 per cent hydrochloric acid with alcohol and after drying at  $105^{\circ}$  gave the following result on analysis:



0.2420 gram substance gave 0.0342 gram  $H_2O$  and 0.0725 gram  $CO_2$   
 0.2331 gram substance gave 0.1422 gram  $BaSO_4$  and 0.1384 gram  $Mg_2P_2O_7$

For  $C_{12}H_{22}O_{41}P_{10}Ba_5 = 1818$

Calculated C, 7.92, H, 1.21, P, 17.05, Ba, 37.73 per cent

Found I {C, 8.02, H, 1.58, P, 16.66, Ba, 36.66 per cent  
 II {C, 8.17, H, 1.58, P, 16.55, Ba, 35.90 per cent

As repeated precipitations did not alter the composition it was undoubtedly a homogeneous compound. The barium was found to be too low, but as previously remarked it is difficult to obtain these amorphous salts in absolutely pure form. The percentage of the base combined with the acid is apt to vary more or less, depending upon conditions. The analysis of the free ester leaves no doubt but that the substance was the compound in question.

#### *Preparation of the free di-inosite-tri-pyrophosphoric acid ester*

The purified dry barium salt (1.5 grams) was suspended in water, decomposed with slight excess of sulphuric acid, the barium sulphate removed and the solution precipitated with copper acetate. The copper salt was decomposed and the free ester obtained in exactly the same way as described for the di-pyrophosphoric ester.

In this case also the copper sulphide could be precipitated only after the solution had been acidified with hydrochloric acid. For the removal of the hydrochloric acid the filtrate was evaporated several times in vacuum with water and finally dried in vacuum over sulphuric acid and potassium hydroxide. The product, like the previous compound, was a thick light amber colored syrup. For analysis it was dried at  $105^\circ C$ .

0.1607 gram substance gave 0.0466 gram  $H_2O$  and 0.0744 gram  $CO$

0.1083 gram substance gave 0.1030 gram  $Mg_2P_2O_7$

For  $C_{12}H_{22}O_{41}P_{10} = 1142$

Calculated C, 12.60, H, 2.80, P, 27.14 per cent

Found C, 12.62, H, 3.24, P, 26.51 per cent

#### *Properties of the di-inosite tri-pyrophosphoric ester*

The properties of this ester agree in the main with those mentioned of the di-pyrophosphoric ester. The concentrated solution of the ester forms a thick light amber colored syrup which on longer drying in desiccator becomes brittle and hygroscopic. The aqueous

solution is of strong acid reaction and pleasant acid taste. The precipitates produced with calcium, magnesium, silver and iron salts are identical with those given by the di-pyrophosphoric ester.

Barium chloride produces at once a white precipitate sparingly soluble in acetic but readily soluble in dilute hydrochloric and nitric acids.

Ordinary molybdate solution produces a white precipitate which does not turn yellowish in color, being in this respect identical with phytic acid.

Neutral molybdate solution causes at first a voluminous white precipitate which redissolves almost immediately. The addition of a few drops of the ordinary acid molybdate to this solution and scratching with a glass rod causes the separation of long white needle-shaped crystals. The crystals and the precipitate caused by the ordinary molybdate solution are readily soluble in ammonia.

On drying at  $105^{\circ}$  the substance turns very dark in color.

Lack of material prevented the hydrolysis of this ester and the isolation of inositol as one of the products of decomposition had therefore to be omitted.



# THE INFLUENCE OF SODIUM TARTRATE UPON THE ELIMINATION OF CERTAIN URINARY CONSTITUENTS DURING PHLORHIZIN DIABETES

By FRANK P UNDERHILL

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Connecticut )

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In recent communications by Baer and Blum<sup>1</sup> it is pointed out that the subcutaneous administration of a series of organic compounds containing two carboxyl groups exercises a remarkable inhibitory influence upon the elimination of urinary nitrogen and dextrose in dogs with phlorhizin diabetes. Among the substances possessing this property may be mentioned glutaric and tartaric acids and their salts.

The results obtained by these authors are so striking and of such fundamental importance in the interpretation of the mechanism of phlorhizin diabetes that a reinvestigation of the problem seemed desirable. Accordingly experiments have been planned similar to those of Baer and Blum the details of which are appended.<sup>2</sup> The investigation has corroborated the reported results but has yielded an explanation for the phenomena observed which is different from that put forth by Baer and Blum. Our work has been confined to the study of the action of a single compound used by Baer and Blum, namely, sodium tartrate, prepared by neutralization of the racemic crystalline tartaric acid (Kahlbaum and other preparations) with sodium carbonate. Both rabbits and dogs were employed as experimental animals.

<sup>1</sup> Baer and Blum *Hofmeister's Beiträge*, x, p 80, 1907, xi, p 102, 1908, *Arch f exp Path u Pharm*, lxxv, p 1, 1911.

<sup>2</sup> A notice of this investigation was communicated to the Society for Experimental Biology and Medicine, May 15, 1912.

After the completion of our experiments a preliminary account of the influence of glutaric acid on phlorhizin diabetes was reported by A I Ringer<sup>3</sup> In this communication Ringer has entirely failed to confirm the reported results of Baer and Blum with respect to glutaric acid

**METHODS** The general plan of experimentation was similar to that of Baer and Blum Phlorhizin diabetes was established for a preliminary period (usually three days) in the fasting animal, the drug being given subcutaneously once daily in sodium carbonate solution Water was allowed *ad libitum* Urine was collected in twenty-four-hour periods either by compression of the bladder in rabbits or by catheterization in the case of dogs Tartrate administration occurred immediately after the phlorhizin injection and the quantities of tartaric acid specified in the tables were subcutaneously injected subsequent to neutralization with sodium carbonate

In our preliminary trials we repeated the work of Baer and Blum employing rabbits instead of dogs As may be seen from tables 1, 2, 3, and 4, sodium tartrate administered subcutaneously to rabbits with phlorhizin diabetes promptly causes a very decided diminution in the output of total nitrogen and dextrose It will also be observed, however, that urine secretion is greatly diminished and in some of the experiments, of which the appended are a few examples only, was completely inhibited From the data in the first four tables it is evident that suppression of urine is sufficient to account for the very great decrease in the output of the urinary constituents under consideration When the urine secretion was not entirely inhibited we have at times obtained water-clear twenty-four-hour specimens of fair volume in which *no trace of nitrogen or dextrose could be detected*

Experiments with dogs yielded results in accord with those obtained with rabbits as may be seen from the examples cited in tables 5 and 6 In experiment 7, dog 1, table 5, the change in the output of urine and of the urinary constituents under discussion shows a striking similarity to that observed in rabbits Experiment 8, dog 3, table 6, is inserted to show that at times one dog may perhaps be less susceptible to the action of tartrate than other individuals, and that water may be eliminated by the kidney even

<sup>3</sup> Ringer *Proceedings of the Society for Experimental Biology and Medicine*, ix, p 54, 1912

TABLE I

## EXPERIMENT I, RABBIT A

*Male rabbit of 2200 grams received daily subcutaneous injection of 0.25 gram phlorhizin*

DATE 1911	URINE				REMARKS
	Volume	Specific gravity	Total Nitrogen	Dextrose	
November	cc		grams	grams	
14	90	1.040	1.85	2.72	
15	100	1.026	1.60	1.80	
16	100	1.030	1.99	1.43	
17	30	—	0.13	0.00	Subcutaneous injection of 3.0 grams tartaric acid, neutralized with Na <sub>2</sub> CO <sub>3</sub> , in 40 cc water
18	8	—	0.00	0.00	Animal lies in deep coma. Does not respond to stimulation. Heart beat and respiration very slow.
19	0				
20	0				Animal found dead. Bladder empty. No urine secreted for 24 hours.

when the latter is no longer in a condition to normally secrete the organic constituents of the urine. With rabbits exactly analogous conditions may obtain. In other words, a dose of sodium tartrate which in the majority of rabbits or dogs causes suppression of urine may exert only a slight influence in this direction in a small number of individuals. An inspection of the data presented by Baer and Blum points to the same conclusion, and it is possible that the negative results reported by Ringer may be due to the same fact. This seems hardly likely, however, and as a possible explanation of Ringer's failure to corroborate the findings of Baer and Blum we would call attention to the fact that Ringer administered his glutaric acid solutions in three equal doses during the course of the day and in this way failed, perhaps, to overwhelm the capacity of the animal to transform the compound into a harmless derivative.

TABLE 2  
EXPERIMENT 2, RABBIT B

*Female rabbit of 2300 grams received daily subcutaneous injection of 0.25 gram phlorhizin*

DATE 1911	URINE				REMARKS
	Volume	Specific gravity	Total nitrogen	Dextrose	
November	cc		grams	grams	
14	115	1.030	1.66	3.58	
15	75	1.036	1.58	1.39	
16	100	1.030	1.82	1.28	
17	55	—	0.06	0.17	Subcutaneous injection of 30 grams tartaric acid, neutralized with $\text{Na}_2\text{CO}_3$ , in 40 cc water
18	10	—	0.05	0.00	
19	0				Animal lies in cage, cannot stand. Has no muscular control. Heart beat and respiration are greatly accelerated.
20	0				Found dead. No urine secreted for twenty-four hours.

From the data in tables 1, 2, 3, and 4, it is evident that sodium tartrate produces its unique influence upon the elimination of urinary nitrogen and dextrose in phlorhizinized animals by causing a partial or complete suppression of urine. In order to determine further the correctness of this conclusion, sections of the kidneys were preserved and sent to Prof. H. Gideon Wells of the University of Chicago to whom I am greatly indebted for the examination of the tissues. In his report Professor Wells says in part, "The greater part of the epithelium of the convoluted tubules is entirely necrotic, and most of the tubules, almost all, in fact, are occluded by large hyaline and granular casts, frequently containing more or less hemoglobin. It is easy to understand that such a kidney could not secrete. It is quite as severe a change as I have ever seen in experimental nephritis. There is very little difference between any

TABLE 3  
EXPERIMENT 3, RABBIT C

*Female rabbit of 2200 grams received daily subcutaneous injection of 0.25 gram phlorhizin*

DATE 1911	URINE				REMARKS
	Volume	Specific Gravity	Total nitrogen	Dextrose	
November	cc		grams	grams	
21	85	1 026	0 95	1 88	Drank 80 cc water
22	75	1 030	1 30	1 16	Drank 45 cc water
23	75	1 030	1 18	0 87	Drank 50 cc water
24	30	1 012	0 00	0 00	Subcutaneous injection of 1 75 grams tartaric acid, neutralized with $\text{Na}_2\text{CO}_3$ , in 30 cc water Drank 140 cc water
25	40	1 015	0 07	0 00	Would not drink Partial prolapse of uterus Animal killed On autopsy all organs appeared normal except the kidneys which seemed very pale and soft

of the specimens, and that only in degree The glomerules show almost no change beyond an occasional small hemorrhage " Concerning the histology of the dog kidneys Professor Wells reported that vacuolization was much more prominent than necrosis

The histological picture therefore coincides with the other data In their investigation Baer and Blum apparently made no histological study of the kidneys, hence their failure to fully recognize the cause of the diminished excretion of the urinary constituents It is only fair, however, to add that the suspicion of a kidney factor must have entered into their ideas since they have recorded some experiments with this point in mind <sup>4</sup> But they tested the secretory power of the kidney by means of inorganic salts only and

<sup>4</sup> Baer and Blum *Arch f exp Path u Pharm*, lxx, p 1, 1911



TABLE 4

## EXPERIMENT 4, RABBIT D

*Female rabbit of 2200 grams received daily subcutaneous injection of 0.25 gram phlorhizin*

DATE 1911	URINE				REMARKS
	Volume	Specific gravity	Total nitrogen	Dextrose	
November	cc		grams	grams	
21	125	1.024	1.12	2.75	Drank 70 cc water
22	60	1.036	1.17	1.28	Drank 25 cc water
23	50	1.040	0.92	1.19	Drank 45 cc water
24	18	—	0.05	0.00	Subcutaneous injection of 20 gram tartaric acid, neutralized with $\text{Na}_2\text{CO}_3$ , in 40 cc water. Animal drank 125 cc water.
25	2 drops	—		0.00	Urine clots in jelly-like mass. Would not drink.
26	3	—		0.00	Would not drink.
27	0				At 5 p.m. animal was seized with convulsions and died in few moments. At autopsy all organs appeared normal except the kidneys which were pale and soft.

report no change in the elimination of these compounds and therefore conclude that kidney secretory factors are not primarily accountable for their results. However, it does not necessarily follow that in a given form of nephritis inorganic salts alone may not be eliminated, nor is it fair to assume that, because one substance may be excreted, a second compound of an entirely different chemical nature will behave in the same manner. If these results of Baer and Blum with inorganic salts are accepted our results indicate the correctness of our contention. It is not our intention, however, at this time to enter more deeply into the conditions attendant upon tartrate nephritis but rather to indicate that a nephritic

TABLE 5  
EXPERIMENT 7, DOG 1*Full-grown bitch of 9 kilos received daily subcutaneous injection of 1.5 grams phlorhizin*

DATE 1912	URINE				REMARKS
	Volume	Specific gravity	Total nitrogen	Dextrose	
February	cc		grams	grams	
6	220	1.070	6.42	32.82	D N ratio = 5.11 Animal drank 120 cc water
7	415	1.074	11.70	43.33	D N ratio = 3.70 Animal drank 250 cc water
8	600	1.053	11.98	40.06	D N ratio = 3.34 Animal drank 70 cc water
9	125	1.008	0.16	0.00	8.0 grams tartaric acid, neutralized with Na <sub>2</sub> CO <sub>3</sub> , were subcutaneously injected, dissolved in 50 cc water
10	10	—	0.075	0.25	Animal has lost control of muscles and lies in cage Vomits any water given At the close of this day it was apparent that dog would not survive the night Animal was killed by chloroform All organs appeared normal

condition must be taken into consideration in the discussion of the results reported by Baer and Blum. Some of the factors of tartrate nephritis will be detailed in a subsequent communication shortly to appear, the work of which has already been completed.

The present problem has also been attacked from another standpoint. It is quite conceivable that phlorhizin, acting, presumably, specifically upon the kidney structure, may render the latter unusually sensitive to tartrate action and therefore that the combina-

TABLE 6

## EXPERIMENT 8, DOG 3

*Full-grown bitch of 11.0 kilos received daily subcutaneous injection of 1.6 grams phlorhizin*

DATE 1912	URINE				REMARKS
	Volume	Specific gravity	Total Nitrogen	Dextrose	
March	cc		grams	grams	
5	240	1.070+	4.44	31.05	D N ratio = 6.90 Animal drank 370 cc water
6	240	1.070+	8.11	29.44	D N ratio = 3.63 Animal drank 250 cc water
7	307	1.060	8.79	29.75	D N ratio = 3.30 Animal drank 290 cc water
8	442	1.035	3.05	12.88	Subcutaneous injection of 10.0 grams tartaric acid, dissolved in 50 cc fluid and neu- tralized with Na <sub>2</sub> CO <sub>3</sub>
9	335	1.050	7.68	15.12	After injection ani- mal vomited repeat- edly Could not drink because of vomiting
10	209	1.045	1.27	4.06	
11	240	1.016	1.08	1.02	Animal developed ab- cess at site of injec- tion In a weak con- dition Vomits con- tinually Killed with chloroform At au- topsy all organs ap- peared normal

tion of the two drugs may bring about changes in the kidney that neither alone could accomplish. If, however, sodium tartrate has a specific action upon kidney secretion this should be manifested by exclusion of the phlorhizin effect, all other conditions remaining unchanged. We have endeavored to compass this result and in order to measure the extent of kidney secretion (in the absence of

TABLE 7

## EXPERIMENT 5, RABBIT E (Control)

*Male rabbit of 2200 grams No phlorhizin was given throughout experiment*

DATE 1911	URINE					REMARKS
	Volume	Specific gravity	Total nitrogen	Creat- inine	Creatine	
November	cc		grams	milli- grams	milli- grams	
22	105	1 012	0 85	84	9	Animal drank 150 cc water
23	65	1 022	0 81	87	34	Animal drank no water
24	70	1 020	1 22	78	63	Animal drank 25 cc water
25	30	1 015	0 12	Trace, too small to es- timate	Trace, too small to es- timate	Subcutaneous injection of 30 grams tartaric acid, neutralized with Na CO <sub>3</sub> , in 40 cc water Animal drank 30 cc water No symptoms followed injection
26	less than 1 cc	—	—	—	—	Animal drank 110 cc water
27	0	—	—	—	—	Animal drank 60 cc water Animal appears normal except that head is rotated to to the right During morning had one convulsion Recovered
28						Found dead At autopsy peritoneal cavity contained 30 cc of clear fluid that readily clotted The kidneys presented an injected appearance All other organs seemed normal Bladder contained no urine

TABLE 8

## EXPERIMENT 6, RABBIT F (Control)

*Female rabbit of 2200 grams No phlorhizin was given throughout experiment*

DATE 1911	URINE					REMARKS
	Volume	Specific gravity	Total nitrogen	Creat- inine	Creatine	
November	cc		grams	milli- grams	milli- grams	
22	115	1 011	0 74	90	21	Animal drank 90 cc water
23	70	1 020	0 97	111	39	Animal drank no water
24	60	1 025	0 90	84	48	Animal drank 50 cc water
25	32	1 015	0 15	Trace, but too small to es- timate	Trace, but too small to es- timate	Subcutaneous injection of 30 grams tartaric acid, neutralized with $\text{Na}_2\text{CO}_3$ , in 40 cc water No symptoms followed injection Animal drank 60 cc water
26	less than 5 cc	—	—	—	—	Animal drank 25 cc water Peculiar position of head similar to that of Rabbit E
27	0	—	—	—	—	Animal in light coma Killed with chloroform Kidneys were very pale and soft All other organs were apparently normal Bladder was empty

glycosuria), have noted the excretion of urine and total nitrogen as usual and, in addition, the elimination of creatinine and creatine, the latter compound being constantly present in the urine of our fasting animals. In the data presented in tables 7 and 8 the water intake was observed in order to discover whether diminished volume of urine could be accounted for by lack of water consumption. It will be seen that there is no strict correlation between the intake and the output of water in the tables mentioned, a fact which also applies to the data contained in all the other tables. From the

experiments with fasting rabbits, given subcutaneous injections of tartrate only, it is evident that the result presented is one induced specifically by the tartrate and apparently bears little or no relation to the application of phlorhizin

The histological examination revealed no recognizable differences in the kidney changes of specimens taken from animals receiving both phlorhizin and tartrate and from those to whom only tartrate had been administered. In his partial report above, Professor Wells says, "There is very little difference between any of the specimens, and that only in degree." The kidneys taken from animals represented in tables 7 and 8 were sent to Professor Wells mixed in the lot excised from animals having had an injection of both phlorhizin and tartrate. From these observations it is apparent that sodium tartrate alone is capable of inducing a particularly severe form of nephritis when subcutaneously introduced into rabbits and dogs.

Our conception of the mechanism responsible for the diminution in urinary constituents as reported by Baer and Blum also furnishes a reasonable explanation for the toxicity of tartaric acid observed by these investigators. In their last paper<sup>6</sup> upon the subject tartaric acid action is discussed as follows, "Weiterhin besass die Saure eine erhebliche Giftigkeit. Ohne bemerkenswerte Symptome starb die Mehrzahl unserer Hunde kurz nach Beendigung, einzelne Tiere sogar vor Beendigung des Versuchs. Immerhin glauben wir diese giftige Wirkung als etwas Akzidentelles auffassen zu dürfen, nicht als die Ursache des Einflusses auf Zucker-, Stickstoff- und Acidosekorperausscheidung."

#### SUMMARY

The observation of Baer and Blum that sodium tartrate subcutaneously injected may greatly diminish the output of nitrogen and dextrose in the urine of phlorhizinized dogs has been substantiated by the results of our investigation on the subject, but we differ from these authors in the interpretation of the phenomena provoked.

Our experience shows that *sodium tartrate subcutaneously administered to phlorhizinized rabbits and dogs induces disintegrative changes*

<sup>6</sup> Baer and Blum *Arch f exp Path u Pharm*, lxxv, p 16, 1911

*in the kidney tubule sufficient to account for the lessened elimination of urinary nitrogen and dextrose, observed by Baer and Blum*

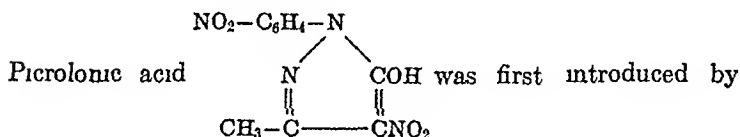
Under strictly comparable experimental conditions similar results may be obtained in animals that have not received phlorhizin, thus demonstrating that sodium tartrate acts specifically in this direction and that phlorhizin probably contributes little or nothing to the detrimental influence under discussion

# PICROLONATES OF THE MONOAMINO-ACIDS

By P A LEVENE AND DONALD D VAN SLYKE

(From the Laboratories of the Rockefeller Institute for Medical Research,  
New York )

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Knorr<sup>1</sup> as a precipitant for organic bases Steudel<sup>2</sup> applied it to the hexone bases obtained by hydrolysis of proteins Later Mayeda<sup>3</sup> described also the picrolonates of the aromatic amino-acids, tryptophane and phenylalanine The latter was particularly distinguished by its slight solubility in water In the course of a hydrolysis we found this picrolonate of so much assistance in the isolation of phenylalanine that we attempted to make the picrolonates of some of the other monoamino-acids, with the idea that they also might prove useful in the separations that are necessary in carrying out protein hydrolyses We found that all the natural monoamino-acids, with the exception of the pyrrolidine acids, proline, and oxyproline, gave beautiful crystalline, definite salts with picrolonic acid, most of them being fairly insoluble in cold water When this work had been completed and the report sent in to the Secretary of the Society for Experimental Biology and Medicine for the meeting of May 15, an article by Abderhalden and Weil<sup>4</sup> appeared describing picrolonates of alanine, glycocoll, and inactive leucine This

<sup>1</sup> Ber d deutsch chem Gesellsch, xxx, p 909, 1897

<sup>2</sup> Zeitschr f physiol Chem, xxxviii, p 219

<sup>3</sup> Ibid, li, p 261

<sup>4</sup> Ibid, lxxviii, p 150



paper antedates our report in demonstrating that the ordinary monoamino-acids form crystalline picrolonates. As, however, our technique was somewhat different from that of Abderhalden and Weil, yielded products of constant molecular composition, and our work included the preparation and study of a larger series of picrolonates, we decided to publish it in the present paper.

Abderhalden and Weil added an alcoholic solution of picrolonic acid to the concentrated water solution of the amino-acids. Crystalline products were obtained, but, in the case of glycocoll and alanine, when an excess of the amino-acid was present the product contained more than 1 molecule of amino-acid. We never met this difficulty. Our procedure is as follows. The amino-acid and picrolonic acid in molecular proportions, or with amino-acid in excess, are dissolved in a minimum amount of boiling water. On cooling, and usually while the solution is still warm, the picrolonate crystallizes. The product obtained was of normal composition, containing one molecule each of amino-acid and picrolonic acid, in every case except those of *d*-alanine, *dl*-serine, and *d*-glutaminic acid. These salts showed a tendency to carry down an excess of picrolonic acid. The latter, however, was readily removed by shaking out the pulverized salt with ether. The salts all show definite crystalline form, which in some cases is quite characteristic. Isoleucine, for example, crystallizes in long slender prisms, while *l*-leucine forms rhomboids. If one makes a picrolonate of the so-called natural leucine from proteins, which is a mixture of the two, the two types of crystals can be seen together under the microscope. Most of the picrolonates decompose when they melt, and many of them have decomposing points too indefinite to be of value for characterizing them. Others, however, melt or decompose quite sharply.

For separating mixtures of amino-acids by means of their picrolonates two methods are possible. One can either transform all the amino-acids into picrolonates, and recrystallize them, or one can add to the mixture of amino-acids only enough picrolonic acid to combine with that forming the most insoluble picrolonate. We have not tried out either method exhaustively but we have found that the latter gives good results in separating phenylalanine from glutaminic and aspartic acids.

For determining the molecular proportions in which amino-acid and picrolonic acid are present in a given salt the most direct method is determination of the proportion of amino nitrogen present. The determination is conveniently carried out as follows. 0.1 – 0.2 gram of picrolonate is dissolved in boiling water to which 1 cc. of 10 per cent hydrochloric acid is added. The solution is cooled, and the picrolonic acid which crystallizes out is filtered off, the filtrate being received into an evaporating dish. The crystals are washed a few times, and the filtrate concentrated on the water bath, and used for determination of amino nitrogen by the nitrous acid method of Van Slyke<sup>5</sup>. The removal of picrolonic acid is necessary, because, even when the solubility of the picrolonate permits using it directly for the determination, the picrolonic acid set free in the nitrous acid mixture makes a gelatinous mass which renders the determination difficult.

The free amino-acids can be readily and quantitatively regained from the picrolonates by boiling the latter in water containing an excess of  $\frac{N}{T}$  sulphuric acid, filtering off the picrolonic acid which crystallizes from the cooled solution, and extracting with ether the small amount of picrolonic acid remaining in solution. The solution is heated on the water bath, and the sulphuric acid precipitated with an equivalent of titrated barium hydrate solution. On evaporating the filtrate from the barium sulphate the free amino-acid is left in pure condition as a residue.

In the following table is given a list of the picrolonates which we have prepared, with some of the more important constants of each. The picrolonates are arranged in inverse order of their solubility.

<sup>5</sup> This *Journal* ix, p 185, 1911

TABLE I

PICROLONATE OF	EMPIRICAL FORMULA	MOLECULAR WEIGHT	PERCENTAGE OF AMINO-ACID IN PICROLONATE	GRAMS SOLUBLE IN 100 CC H <sub>2</sub> O AT 20-23°	AMINO-ACID IN PICROLONATE DISSOLVED IN 100 CC H <sub>2</sub> O	MELTING OR DECOMPOSITION POINT (UNCORR)	SPECIFIC ROTATION $[\alpha]_D^{20}$
<i>dl</i> -Phenylalanine	$C_{15}H_{15}N_2O_7$	429.2	38.4	0.12	0.05	212° (decomp)	-
Tyrosine	$C_{15}H_{13}N_2O_8$	445.2	40.7	0.29	0.12	Blackens 260°	-
<i>l</i> -Phenylalanine*	$C_{15}H_{13}N_2O_7$	429.2	38.4	0.34	0.13	208° (decomp)	+ 30.1°
<i>dl</i> -Leucine	$C_{16}H_{21}N_2O_7$	395.2	33.1	0.53	0.17	Indefinite over 140°	-
<i>l</i> -Leucine	$C_{16}H_{21}N_2O_7$	395.2	33.1	0.55	0.18	Indefinite about 150°	+ 19.6°
<i>d</i> -Isoleucine	$C_{16}H_{21}N_2O_7$	395.2	33.1	0.58	0.19	170°	+ 32.8°
<i>dl</i> -Valine	$C_{15}H_{19}N_2O_7$	381.2	30.7	0.81	0.25	Indefinite over 150°	-
<i>dl</i> -Serine	$C_{15}H_{19}N_2O_8$	369.2	28.4	0.98	0.28	Decomp 265°	-
Glycocoll	$C_{12}H_{13}N_2O_7$	339.2	22.1	0.99	0.22	214° (decomp)	-
<i>dl</i> -Alanine	$C_{13}H_{15}N_2O_7$	353.2	25.2	1.01	0.25	216° (decomp)	-
<i>d</i> -Valine*	$C_{15}H_{19}N_2O_8$	381.2	30.7	1.20	0.37	170°-180°	+ 29.2°
<i>d</i> -Alanine	$C_{13}H_{15}N_2O_8$	353.2	25.2	1.61	0.41	214° (decomp)	+ 12.4°
<i>dl</i> -Aspartic acid	$C_{14}H_{17}N_2O_9$	397.2	33.5	1.69	0.57	Blackens 130°	-
<i>dl</i> -Glutamic acid	$C_{15}H_{19}N_2O_9$	411.2	35.8	2.37	0.85	184° (decomp)	-
<i>d</i> -Glutamic acid	$C_{15}H_{19}N_2O_9$	411.2	35.8	easily sol		184° (decomp)	+ 8.5

\* The phenylalanine available was 2% per cent racemized consequently solubility and melting point may be slightly different in the optically pure substance. The rotation is calculated for optically pure substance. The  $d$  value was 19.8 per cent racemic. All rotations were taken with alcoholic solutions except that of  $d$  glutamic acid of which aqueous solution was used.

## EXPERIMENTAL

*dl-Alanine* One gram of alanine and 2.6 grams of picrolonic acid (0.9 molecule) were dissolved in 20 cc of boiling water. The salt crystallized while the solution was still hot. The yield was 3.2 grams. The crystals were long slender prisms. They melt at 216° with decomposition.

ANALYSIS 0.200 gram substance, 13.40 cc nitrogen at 22°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.96	3.78

*d-Alanine* 0.445 gram of *d*-alanine and 1.32 grams (1 molecule) of picrolonic acid were dissolved in 15 cc of water. The substance crystallized while the solution was still warm, forming long slender prisms. Yield, 1.3 grams. Analysis showed only 3.10 per cent of amino nitrogen instead of the theoretical 3.96. The substance was pulverized and shaken up with ether. It now gave the following figures

ANALYSIS 0.200 gram substance 13.70 cc N at 20°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.96	3.90

The substance melts at 214° with decomposition.

ROTATION IN ALCOHOLIC SOLUTION Substance, 0.0496 gram, solution, 0.6141 gram, concentration, 8.07 per cent, specific gravity, 0.82, rotation in 0.5 dm tube = +0.41°

$$[\alpha]_D^{20} = +12.4^\circ \pm 0.3^\circ$$

Abderhalden and Weil give 215° as the melting point, +11.1° as the specific rotation.

*dl-Aspartic acid* 0.67 gram of aspartic acid and 1.32 grams of picrolonic acid (1 molecule) were dissolved in 50 cc of boiling water. The solution was cooled in the ice box and allowed to stand over night for the crystallization to become complete. The crystals were long slender prisms with square ends. The substance blackens at 130°.

ANALYSIS 0.100 gram substance, 6.10 cc N at 20°, 764 mm

	Calculated	Found
NH <sub>2</sub> -N	3.53	3.49

*dl-Glutaminic acid* 0.736 gram of glutaminic acid + 1.32 grams of picrolonic acid (1 molecule) were dissolved in 10 cc of water. Crystallization began only after the solution had been cooled to room temperature and scratched. The first crystals came in tangled threads, the later ones formed in very fine short spindles or needles. Yield, 1.7 grams. The substance softens at 183° and decomposes at 184°. These points are sharply defined.

ANALYSIS 0.200 gram substance, 11.8 cc nitrogen at 20°, 762 mm

	Calculated	Found
NH <sub>2</sub> -N	3.40	3.36

*d-Glutaminic acid* 0.74 gram of pure *d*-glutaminic acid and 1.32 grams of picrolonic acid (1 molecule) were dissolved in about 10 cc of water. On cooling 0.7 gram of crystals separated. On analysis, however, they were found to contain about two molecules of picrolonic acid. They were shaken out with ether, and then gave more nearly correct figures on analysis (3.25 per cent NH<sub>2</sub>-N). The filtrate from this first crop was cooled to 0°, and a second crop separated, which proved to be fairly pure without ether treatment. The substance, like the inactive salt, softens at 183° and melts at 184°. The rotation was taken in water solution.

ANALYSIS 0.100 gram substance, 6.3 cc N at 766 mm, 21°

	Calculated	Found
NH <sub>2</sub> -N	3.40	3.59

ROTATION 0.0562 gram substance, solution, 2.683 grams, concentration, 2.10 per cent, specific gravity 1.01, rotation in 0.5 dm tube = +0.09°

$$[\alpha]_D^{20} = +8.5^\circ \pm 1^\circ$$

*Glycocoll* 0.375 gram glycocoll and 1.32 grams picrolonic acid (1 molecule) were dissolved in 15 cc of boiling water. The salt began to crystallize in rhomboid prisms of very characteristic appearance while the solution was still warm. It was allowed to stand over night and then yielded 1.56 grams of the salt. It melts sharply at 214 to 215° with decomposition.

ANALYSIS 0.200 gram substance, 15.8 cc N at 20°, 762 mm

	Calculated	Found
NH <sub>2</sub> -N	4.12	4.19

Abderhalden found that in alcoholic solution in the presence of an excess of glycocoll a picrolonate was obtained containing two molecules of glycocoll to one of picrolonic acid. We repeated the experiment, using two molecules of glycocoll, but an aqueous solution. The product was a normal salt identical with the above. 0.75 gram of glycocoll and 1.32 gram of picrolonic acid were dissolved in 15 cc of boiling water and cooled. Crystallization began while the solution was still warm, yielding the above described characteristic rhomboids. Yield, 1.54 grams. Melting point, 214-215°.

ANALYSIS 0.200 gram substance, 15.3 cc N at 22°, 750 mm

	Calculated	Found
NH <sub>2</sub> -N	4.12	4.13

In calculating the nitrogen for both the above analyses the results are multiplied by the factor 0.93 in order to correct for the abnormal behavior of glycocoll with nitrous acid, which has been described in a previous paper by one of us.<sup>6</sup>

*d*-Isoleucine 0.35 gram isoleucine, of +37.4° specific rotation in 20 per cent hydrochloric acid, and 0.66 gram of picrolonic acid (0.9 molecule) were dissolved in 15 cc of boiling water. While the solution was still warm the salt began to separate as drops which changed quickly to crystals. The latter were long, slender, six-sided and grouped in stars. The substance has a rather indefinite melting point at 170°, without decomposition.

ANALYSIS 0.100 gram substance, 6.40 cc N at 23°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.54	3.59

ROTATION 0.0672 gram substance, solution in alcohol, 1.039 gram, concentration, 6.47 per cent, specific gravity, 0.82, rotation in 0.5 dm tube = +0.87°

$$[\alpha]_D^{20} = +32.8^\circ \pm 0.3^\circ$$

*l*-Leucine 0.66 gram of *l*-leucine, of +15.2° specific rotation in 20 per cent hydrochloric acid, was dissolved with 1.32 grams (1 molecule) of picrolonic acid in 15 cc of boiling water. While the solution was still hot the salt began to settle out as an oil,

<sup>6</sup> Van Slyke. *This Journal*, 15, p. 185, 1911.

which crystallized at once when stirred with a rod Yield, 1.7 grams The substance begins to soften at about 145° and melts completely at 150° with evolution of a few small gas bubbles

ANALYSIS 0.200 gram substance, 12.4 cc N at 22°, 750 mm

	Calculated	Found
NH-N	3.54	3.45

ROTATION 0.0462 gram substance, solution in alcohol, 0.6390 gram, concentration, 7.23 per cent, specific gravity, 0.813, rotation in 0.5 dm tube = +0.58°

$$[\alpha]_D^{20} = +19.6^\circ \pm 0.3^\circ$$

The salt forms beautiful rhomboid crystals, markedly different from the long slender ones of isoleucine picrolonate

"Natural" Leucine The leucine used was analytically pure, but of +23° specific rotation in 20 per cent hydrochloric acid, and was a typical sample of the mixture of leucine and isoleucine usually obtained from hydrolyzed proteins 0.6 gram of this leucine and 1.32 grams picrolonic acid were dissolved in 20 cc of boiling water While the solution was still warm the salt began to separate out in oil drops which quickly became crystalline when stirred Under the microscope the long slender crystals of isoleucine picrolonate were seen mingled with the rhomboid ones of *l*-leucine picrolonate The substance melts at about 170° without marked decomposition

ANALYSIS 0.2208 gram substance, 14.9 cc N at 19°, 758 mm  
0.1275 gram substance, 0.2280 gram CO<sub>2</sub>, 0.0623 gram H<sub>2</sub>O

	Calculated	Found
C	48.58	48.70
H	5.29	5.35
NH <sub>2</sub> N	3.55	3.55

*dl*-Leucine 1.4 grams of leucine and 2.64 grams of picrolonic acid were dissolved in 30 cc of boiling water The salt separated as an oil while the solution was still warm, but crystallized quickly in six-sided prisms The last crystals separating formed rosettes with spurs a centimeter long The melting point is very indefinite, and dependent upon the manner in which the substance is heated When plunged at once into a bath at 150° or higher

it decomposes instantly, but when heated from 130° upwards it slowly sinters and may not be decomposed until it reaches 188°

ANALYSIS 0.200 gram substance, 12.20 cc N at 20°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.54	3.47

*l*-Phenylalanine Picrolonate One gram of phenylalanine, of rotation  $-26.7^\circ$  in water solution, was dissolved with 1.6 grams of picrolonic acid in boiling water. The salt crystallized while the water was still hot. Under the microscope two types of crystals could be seen, long slender rods clustered in stars, and short rectangular prisms separate or in small aggregates. When the substance was filtered it was seen to consist of an apparent mixture of yellow and orange crystals. The non-homogeneous appearance, however, was merely due to the presence of two types of crystals. The substance melted at 208° with decomposition, gave theoretical results for carbon and hydrogen on combustion, and the following figures for amino nitrogen

ANALYSIS 0.2148 gram substance, 12.3 cc N at 20°, 762 mm

	Calculated	Found
NH <sub>2</sub> -N	3.26	3.27

ROTATION 0.0696 gram substance, solution in alcohol, 1.080 gram, concentration, 6.44 per cent, specific gravity, 0.818, rotation in 0.5 dm tube =  $+0.60^\circ$

$$[\alpha]_D^{20} = +22.8^\circ \pm 0.3^\circ$$

In order to ascertain whether the picrolonate contained a proportion of the optically active phenylalanine different from that of the partially racemized substance from which it was made, a portion of the picrolonate was freed from picrolonic acid and the pure phenylalanine regained as described in the introduction. It showed a specific rotation of  $-26.7^\circ$ , and was therefore identical with that used at the beginning. The rotation of optically pure *l*-phenylalanine being  $-35.3^\circ$  this sample was evidently 25 per cent racemic and 75 per cent active. Dividing the rotation of the picrolonate by 0.75 gives  $30.1^\circ$  as the specific rotation of the picrolonate of pure active *l*-phenylalanine.



*dl-Phenylalanine* 0.83 gram of phenylalanine and 1.32 grams (1 molecule) of picrolonic acid were boiled with 40 cc of water. The salt was so insoluble that complete solution could not be obtained, even in this volume of water. The product was, however, pure. The yield was practically quantitative, 2.04 grams. The substance decomposes quite sharply at 211 to 212°. Examined under the microscope it is seen to consist of only the short rectangular type of prisms.

ANALYSIS 0.1000 gram substance, 5.80 cc N at 20°, 764 mm

	Calculated	Found
NH <sub>2</sub> -N	3.26	3.32

*dl-Serine* 0.26 gram of serine and 0.66 gram of picrolonic acid were dissolved in 5 to 10 cc of water. The salt crystallized in long slender rods while the solution was still warm. It contained, however, an excess of picrolonic acid, as was shown by its low content (2.56 per cent) of amino nitrogen. A second preparation showed identical composition, 2.59 per cent amino nitrogen. The melting point was indefinite at 130 to 140°. After the substance had been shaken out thoroughly with ether, it could be heated to 200° without change. Above 200° it began slowly to darken, and finally decomposed with foaming at 265°. It now gave the following figures:

ANALYSIS 0.1300 gram substance, 7.9 cc N at 23°, 764 mm

	Calculated	Found
NH <sub>2</sub> -N	3.79	3.43

*Tyrosine* 0.91 gram of tyrosine and 1.32 grams of picrolonic acid (1 molecule) were dissolved in 40 cc of boiling water. On cooling the salt crystallized at once in rods grouped together in rosettes. The substance blackens and sinters at 260° without melting or foaming.

ANALYSIS 0.200 gram substance, 10.70 cc N at 22°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.14	3.02

*d-Valine* 1.3 grams of valine and 2.64 grams of picrolonic acid (0.9 molecule) were dissolved in 10 cc of boiling water. The

salt showed a tendency to separate as an oil, which, however, changed to crystals while the solution was still warm. Yield 3.4 grams. The substance melts between 170° and 180°, without decomposition. The melting point is not sharply defined.

ANALYSIS 0.200 gram substance, 12.95 cc N at 23°, 760 mm

	Calculated	Found
NH <sub>2</sub> -N	3.67	3.63

ROTATION 0.0471 gram substance, solution in alcohol, 0.834 gram, concentration, 5.62 per cent, specific gravity, 0.82, rotation in 0.5 dm tube = +0.54°

$$[\alpha]_D^{20} = +23.4^\circ \pm 0.2^\circ$$

The free valine was regained from 0.5 gram of the picrolonate and its rotation determined. substance, 0.1166 gram, solution in 20 per cent hydrochloric acid, 2.782 grams, concentration, 4.19 per cent, specific gravity, 1.10, rotation in 2 dm tube = +2.13°

$$[\alpha]_D^{20} = +23.1^\circ \pm 0.1^\circ$$

The valine from which the picrolonate was made had been obtained from a protein hydrolysis and showed practically the same rotation. Taking +28.8° as a rotation of optically pure *d*-valine, the sample used is calculated to contain 19.8 per cent of racemic valine and 80.2 per cent of the active. Dividing the rotation observed for the picrolonate by 0.802 gives +29.2° as the rotation of the picrolonate of the pure *d*-valine.

*dl*-Valine 0.60 gram of Kahlbaum's valine and 1.32 grams of picrolonic acid (1 molecule) were dissolved in 15 cc of boiling water. Crystallization began while the solution was still warm. Yield, 1.84 grams. The substance melts at once with vigorous decomposition if plunged into a bath of 150°, if heated from below 130° upwards it gradually darkens and sinters, and may not decompose until it reaches 220°.

ANALYSIS 0.200 gram substance, 13.0 cc N at 21°, 754 mm

	Calculated	Found
NH <sub>2</sub> -N	3.67	3.65

*Solubility Determinations*

For the determination of the solubility in water, 0.4 gram of picrolonate, finely ground, was shaken 4-5 hours with 15 cc of water at room temperature, which varied between 20 and 23°. The solutions were filtered, and 10 cc portions of the filtrate evaporated to dryness in weighed glass dishes. The results are given in Table 1.

For the determination in alcohol the same technique was employed, except that only 8 cc of the solvent were used and only 5 cc evaporated. The following picrolonates are readily soluble in cold alcohol: those of *D*-alanine, *D*-isoleucine, *L*-leucine, *DL*-leucine, tyrosine, and *L*-phenylalanine. *D*-Valine and *DL*-valine dissolve readily when alcohol is warmed slightly. The following solubilities in absolute alcohol were determined quantitatively.

TABLE 2

PICROLONATE OF	GRAMS SOLUBLE IN 100 CC ALCOHOL AT 20 TO 23°
<i>DL</i> -Phenylalanine	0.81
<i>DL</i> -Aspartic acid	1.16
<i>DL</i> -Alanine	2.88
<i>DL</i> -Glutamic acid	3.86
<i>L</i> -Phenylalanine	5.71

The *L*-phenylalanine used was that previously described, and contained 25 per cent of the racemic substance. Although the figures cannot, therefore, be regarded as strictly accurate, they show that the picrolonate of *L*-phenylalanine is several times more soluble in absolute alcohol than that of the *DL*-phenylalanine. By utilizing this difference in solubility *L*- and *DL*-phenylalanine can be partially separated. A mixture of three parts active and five parts inactive picrolonate was shaken with twenty parts of alcohol. Half of the substance was dissolved. Its rotation showed that it was 75 per cent active. The residue was absolutely inactive.

*Separation of Phenylalanine and Aspartic Acid*

In 20 cc of boiling water were dissolved 0.75 gram of phenylalanine (specific rotation  $-27^\circ$ ), 1.25 grams (1 molecule) picro-

ionic acid, and 0.7 gram of *dl*-aspartic acid. The solution was cooled and allowed to stand two hours for crystallization to become complete. The picrolonate was then filtered off and washed with cold water. It weighed 1.76 grams, equivalent to 0.678 gram of phenylalanine. Analysis showed it to be the pure phenylalanine picrolonate.

ANALYSIS 0.1354 gram substance,  $\text{CO}_2$ , 0.2645 gram,  $\text{H}_2\text{O}$ , 0.0555 gram

	Calculated	Found
C	53.12	53.13
H	4.46	4.63

In a similar manner we were able to separate a mixture of glutamic acid and phenylalanine which we encountered in a hydrolysis of hemoglobin that will shortly be the subject of another article. The use of picrolonic acid in the isolation of phenylalanine by the ester method decidedly improves the accuracy of the determination. Because of the crystalline nature of the picrolonates and the insolubility of many of them, it is possible that with their assistance the number of amino-acids which can be obtained from hydrolyzed proteins without esterification may be increased.



# PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

## THIRD PAPER

### FURTHER ABSORPTION EXPERIMENTS WITH ESPECIAL REFERENCE TO THE BEHAVIOR OF CREATINE AND CREATININE AND TO THE FORMATION OF UREA

By OTTO FOLIN AND W. DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston)

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The hypothesis that the nitrogenous digestion products are resynthesized into albuminous materials while passing through the mucous membrane of the intestine and therefore cannot be detected in the blood was originally advanced to explain the absence of peptones in the blood during digestion. The subsequent discovery that protein digestion normally proceeds further than to the peptone stage and results in the formation of amino-acids suggested another explanation of the earlier negative results. As the attempts to find the amino-acids in the blood were no more successful than had been the earlier attempts to find the peptones, the hypothesis of the immediate resynthesis of the digestion products into albuminous substances was revived. Kutscher adopted it<sup>1</sup> and Abderhalden has become more and more positive that it represents the only adequate explanation of the negative results obtained by him and his coworkers in their search for peptones and amino-acids in the blood. Since the resynthesis hypothesis is based on negative findings alone it has no value after the presence in the blood of amino-acids absorbed from the intestine has been positively demonstrated. In our first paper<sup>2</sup> we showed that it is possible by means of our new analytical methods<sup>3</sup> to trace urea, glycocoll and pancreatic amino-acid mixtures not only into the blood, but

<sup>1</sup> *Zeitschr f physiol Chem*, xxxiv, p 529, 1902

<sup>2</sup> *This Journal*, xi, p 87, 1912

<sup>3</sup> *Ibid*, xi, pp 493-536, 1912

also into the general tissues of the body. Moreover, the increases in the non-protein nitrogen of the blood and muscles which we have obtained in absorption experiments are large enough to account for practically all the nitrogenous material absorbed from the intestine. We do not claim to account exactly or quantitatively for all that has been absorbed. It is entirely possible that different tissues absorb different amounts from the blood, and we have therefore omitted all calculations tending to show that all the absorbed material is present in the animal in non-protein form. The results obtained indicate, however, that practically all the absorbed nitrogen can be accounted for, so that for the present at least the hypothesis of immediate protein synthesis in the walls of the intestine must be regarded as superfluous and untenable.

The protein regeneration idea served only as an explanation of the failure to find the absorbed amino-acids, it was anything but an explanation of the formation of those amino-acids in the intestine, it was inconsistent with the rapid urea elimination after protein feeding, and it necessarily presupposed that in chemical composition the serum proteins are very variable, whereas, on the contrary, they appear to remain decidedly uniform in composition.

As an explanation of the failure to find peptones or amino-acids in the blood when they should be there in unusual amounts, the immediate deamination hypothesis therefore seemed more plausible than the protein regeneration doctrine, and was usually accepted as a working hypothesis by those who could not accept the older theory of protein synthesis.

The supposedly high ammonia content of the portal blood, and the results reported by Cohnheim<sup>4</sup> on the production of ammonia and volatile bases accompanying the disappearance of peptone and amino-acids from the digestive tract of fishes (when isolated and suspended in blood or salt solutions) pointed certainly in the direction of deamination. The experiments of Jacobi<sup>5</sup> and of Lang<sup>6</sup> on the deamination of amino-acids by tissue extracts

<sup>4</sup> *Zeitschr f physiol Chem*, li, p 239, 1909, lvi, p 181, 1909, lxxvi, p 293, 1912. In his last paper Cohnheim reports only the finding of ammonia and no amino-acids.

<sup>5</sup> *Zeitschr f physiol Chem*, xlv, p 149, 1900.

<sup>6</sup> Hofmeister's *Beiträge*, v, p 321, 1904.

seemed to furnish direct evidence of the presence of deaminizing ferments in the intestine and liver, and helped therefore materially to strengthen the theory of immediate deamination as the characteristic feature of intermediary protein catabolism. Folin's theory of protein metabolism is based on the same conception. Folin did not commit himself to the localization of the deamination process in the intestine and liver, though he believed that those organs were chiefly responsible for the ammonia and urea formations.

The immediate deamination theory failed of course to explain why the non-protein nitrogen did not increase in the blood during active absorption. The adherents of that theory had to content themselves with the "calculations" of Bergmann and Langstein<sup>7</sup> and others that the speed of the blood was so great as to remove the urea practically as fast as it was formed. In such an application of these calculations it is tacitly assumed that the excretory capacity of the kidneys is quite as efficient as the absorptive capacity of the intestine, for if such were not the case, the volume and speed of the blood stream in the mesenteric and portal circulation could not prevent a temporary accumulation of the absorbed digestion products in the blood.

The results reported in our first paper indicated that the deaminizing power ascribed to the intestine and liver is by no means adequate to prevent the accumulation of amino-acids in the blood, and in our second paper<sup>8</sup> we showed that no specialized deamination process is located in the intestine, and that the ammonia in the portal blood is very small in amount and represents the absorption of ammonia produced in the intestinal lumen, chiefly by putrefactive bacteria.

The results reported in our first two papers are surprising in so far as they failed to reveal any urea formation at all except when ammonia was present in the material used for absorption. This result might be regarded as inconsistent with the fact shown by ordinary feeding experiments that the urea elimination is rapidly increased when protein or amino-acids are fed to men or to animals. The inconsistency is, however, more apparent than real. The "rapid urea elimination" after the intake of nitrogenous food is

<sup>7</sup> Hofmeister's *Beiträge*, vi, p 27, 1904

<sup>8</sup> This *Journal* xi, p 161 1912



measured by hours, whereas in our absorption experiments we were dealing with minutes, and did not carry the experiments beyond one hour. The negative results obtained for the urea during the first stages of absorption prove that the urea formation is not localized in the intestine and liver for the purpose of holding back all amino-acids which are not needed for the rebuilding of body tissues, because if such were the case the increase in the urea contents of the blood should begin practically at once, and should be much greater than the accumulation of amino-acid nitrogen. On the other hand, unless we could show that the urea formation does take place later there would remain a discrepancy between our findings and the facts established by ordinary feeding experiments. In this paper we wish to report results which seem to clear up in a measure the formation of urea from amino-acids.

Before taking up the consideration of experiments specifically planned to throw light on the urea formation we wish to record a few additional absorption experiments with substances which are not adapted for the study of the urea production.

For the sake of brevity we wish to state here certain details which represent the procedure followed in all the experiments recorded in this paper unless otherwise described. (1) For anesthetics we have used ether alone, or ether together with a subcutaneous injection of morphine sulphate, or ether with chloroform (the latter injected, together with the substance investigated, into the intestine). After the first few minutes the ether was administered by means of a tracheal cannula. (2) As soon as the animals were unconscious we laid bare the common carotids to have them ready when wanted and inserted a tracheal cannula in the usual manner. (3) The first sample of blood was then taken from one of the femoral arteries by means of a 2 or 5 cc. pipette, as described in this journal,<sup>9</sup> the gracilis muscle of the opened leg was dissected out, and 5 grams of it was immediately cut fine with scissors and immersed in pure methyl alcohol. (4) The abdomen of the animal was then opened and ligatures applied to the small intestine, one just below the stomach, the other just above the caecum. (5) Unless otherwise stated the kidneys were not ligatured or disturbed. (6) The substance under investigation, dissolved in from 50 to 100 grams of water, was then injected into the small intestine by means of a large syringe and a hypodermic needle and the abdomen promptly closed by means of artery clips. (7) The animal was kept warm by means of an electric stove below the holding frame. (8) The alcoholic blood and tissue extracts were prepared as described.<sup>10</sup> (9) The analytical results are calculated in terms of

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<sup>9</sup> This Journal, vi, p. 527, 1912

<sup>10</sup> Ibid., vi, p. 528, 1912

milligrams of nitrogen per 100 grams of blood or tissue (10) The difference between the amount of nitrogen obtained by washing out the intestine at the end of the experiment and the amount of nitrogen contained in the injected product is given as the amount of substance absorbed A small error may be involved in this procedure, but in order to keep the animals as normal as possible we did not want to wash out the intestine before beginning the experiment We have washed out the intestine at the beginning and have found only a few (30 to 40) milligrams of nitrogen, so the error is certainly of very little significance (11) Unless otherwise stated, no account has been taken of the absorption of water from the intestine or of urine elimination In nearly all of our experiments there was practically no elimination of urine as the bladder remained collapsed, also there was very little water absorption, for the intestine remained full

#### ABSORPTION OF ASPARAGINE

EXPERIMENT 1 Cat 41 (weight, 3163 grams) Last feeding, twenty-four hours before the operation Anesthetic, ether and morphine After ligaturing the blood supply of both kidneys 10 grams of Kahlbaum's asparagine dissolved in about 100 cc of water were injected into the ligatured intestine Asparagine is not very soluble and the absorption was allowed to continue for two hours The following analytical results were obtained

	Milligrams
Asparagine nitrogen injected	2154
Asparagine nitrogen absorbed	1054
I Non-protein nitrogen, control blood	43
II Non-protein nitrogen, jugular vein, twenty-two minutes after injection	49
III Non-protein nitrogen, portal vein, twenty-four minutes after the injection	69
IV Non-protein nitrogen, carotid artery, forty-seven minutes after the injection	52
V Non-protein nitrogen, carotid artery, one hundred and twenty minutes after the injection	62
VI Non-protein nitrogen, portal vein, one hundred and twenty-three minutes after the injection	70
VII Non-protein nitrogen mesenteric vein, one hundred and twenty-nine minutes after the injection	83
VIII Non-protein nitrogen, inferior vena cava, one hundred and thirty-two minutes after the injection	62
Non-protein nitrogen in muscle before the injection	200
Non-protein nitrogen In muscle one hundred and thirty-seven minutes after the injection	235

In the case of asparagine injections it is useless to make urea determinations because the imide group is not sufficiently stable to resist the treatment employed for decomposing urea

The figures recorded in the above experiments are not entirely satisfactory. There is an unmistakable increase in the non-protein nitrogen of the blood and in the muscle extract. Quantitatively the figures are perhaps rather smaller than they should be on the basis of the asparagine absorption. The explanation of the nitrogen deficit is the fact that asparagine is precipitated together with the albuminous materials by the methyl alcohol used as a precipitant. The same difficulty is met with even with such a soluble product as urea, unless the precipitated protein materials are washed with alcohol.

The loss of non-protein nitrogen involved is shown in the experiment recorded below.

**EXPERIMENT 2** To slaughter-house blood containing 30 mgm of non-protein nitrogen per 100 cc was added (1) urea, (2) glycocoll, (3) asparagine, in the proportion of (a) 100, (b) 50 mgm of nitrogen per 100 cc of blood. After precipitating the blood in the usual manner (but without washing the coagulum) with nine volumes of pure methyl alcohol and a little zinc chloride, the following figures were obtained from the filtrates

	ADDED	RECOVERED
	mg	mg
1 a Urea nitrogen	100	82
1 b Urea nitrogen	50	36
2 a Glycocoll nitrogen	100	82
2 b Glycocoll nitrogen	50	36
3 a Asparagine nitrogen	100	44
3 b Asparagine nitrogen	50	36

By varying the analytical procedure to the extent of washing the precipitated albuminous material with fresh portions of methyl alcohol the urea and glycocoll added to the blood can be recovered almost quantitatively, but with asparagine as with creatine or creatinine the loss remains large. Thus when adding 100 mgm of asparagine nitrogen we recovered only 33, and when adding 50 we recovered 21.

In all cases, but particularly in the case of asparagine, the values obtained for the non-protein nitrogen of the blood must therefore be regarded as minimum values so far as the increase due to the absorbed substance is concerned

#### ABSORPTION OF TYROSINE

In view of the insolubility and low nitrogen content of tyrosine it seemed practically hopeless to try to trace its transportation from the digestive tract to the blood and tissues by means of nitrogen determinations. The negative results which have heretofore been obtained by means of Millon's reagent<sup>11</sup> also indicate that tyrosine is absorbed very slowly indeed. Millon's reaction is however not a particularly delicate one for the detection of tyrosine. One part in 10,000 seems to be about the limit for an unmistakable test on the basis of that reaction. A far more delicate color reaction for tyrosine is one recently discovered by Folin and Macallum on the basis of a certain phosphotungstic phosphomolybdic compound, the exact composition of which we do not know, but the practical preparation of which is described elsewhere<sup>12</sup>. By means of this reagent we obtain an unmistakable blue color with solutions containing 1 part of tyrosine in 1 million parts of water. Here we had, therefore, a method by means of which it should be an easy matter to trace tyrosine in the body.

EXPERIMENT 3 Cat 48 (weight, 2530 grams) Last feeding, twenty-four hours before the operation. Anesthetic, ether and morphine. Two grams of tyrosine dissolved in about 125 cc of warm 1 per cent sodium carbonate solution was used for the injection. More tyrosine could not be used for want of a suitable solvent. The usual nitrogen determinations were made on the blood and on muscle extracts only to determine whether any increase in the non-protein nitrogen occurs in the absence of active absorption.

	<i>Milligrams</i>
I Non-protein nitrogen in control blood	39
Urea nitrogen	20
II Non-protein nitrogen, carotid artery, eighty-four minutes after the injection	40
Urea nitrogen	20

<sup>11</sup> H C Bradley *This Journal*, **11**, p 10, 1912

<sup>12</sup> See the next number of this *Journal*

		<i>Milligrams</i>
III	Non-protein nitrogen, carotid artery, one hundred and eighty minutes after the injection	39
	Urea nitrogen	19
IV	Non-protein nitrogen, portal vein, one hundred and eighty-three minutes after the injection	39
	Urea nitrogen	20
V	Non-protein nitrogen, mesenteric vein, one hundred and eighty-six minutes after the injection	39
	Urea nitrogen	19
	Non-protein nitrogen in muscle before the injection	180
	Urea nitrogen	26
	Non-protein nitrogen in muscle one hundred and ninety minutes after the injection	180
	Urea nitrogen	26

For the colorimetric test for tyrosine we evaporated 20 cc of the alcoholic filtrates in the usual manner, and added first about 1 cc of the phosphotungstic-molybdenum reagent, 2 or 3 cc of water, and then solid sodium carbonate in excess (as much as would dissolve). The control extracts of blood and muscle gave an extremely faint reaction, while all the other tests both in the blood and in the second muscle extract were so strong as to unmistakably prove the presence of the tyrosine. It seemed hardly worth while to make the test quantitative because of the small amounts involved, but the reaction is decided enough to be available for quantitative purposes.

#### ABSORPTION OF CREATININE

Creatinine like asparagine adheres so tenaciously to the albuminous precipitate produced on adding alcohol to blood that all of it cannot be recovered. The absorption and general distribution of creatinine is however easily demonstrated by means of the non-protein nitrogen determinations. The colorimetric estimation of creatinine in the alcoholic blood and tissue extracts proved at first rather puzzling. Although our nitrogen determinations showed that our extracts contained enough of the absorbed creatinine to make a colorimetric determination feasible, we failed to get even a sure qualitative test with the alkaline picrate reagent. The negative results could have been due to the transformation of creatinine into creatine, although this seemed unlikely from what we know concerning the metabolism of these two products. Boiling the

extracts with hydrochloric acid or heating the dry residues failed however to yield the creatinine reaction. The true explanation was found on adding creatinine directly to alcoholic blood extracts. The negative results obtained are due to the presence in such extracts of some inhibiting substance. Fortunately that substance is soluble in ether and in chloroform. The fact that muscle and blood contain substances which interfere with the colorimetric determination of creatinine doubtless invalidates at least some of the creatine and creatinine determinations recorded in the literature. The creatinine determinations of Noel Paton<sup>13</sup> in goose flesh, for example, were made under conditions which precluded his finding any creatinine which might have been present in his muscle extracts. To test for creatinine or to determine it we have therefore worked out the following procedure. To the alcoholic blood or tissue extract in an evaporating dish add 0.1 or 0.2 gram of picric acid (in methyl alcohol solution) and evaporate to dryness. Wash the residue several times with chloroform saturated with picric acid and, finally, once with pure chloroform. Then add a little water and alkali in the usual manner and make up to a suitable volume (usually 25 or 50 cc.)

As a standard for such creatinine determinations pure creatinine solutions are rather more satisfactory than the half-normal potassium bichromate solutions originally recommended by Folin. Creatinine keeps indefinitely in tenth-normal hydrochloric acid solutions, and since creatinine can now easily be prepared in pure condition the chief reason for using the bichromate as a standard no longer holds. One gram of creatinine dissolved in a liter of tenth-normal acid can be used for several hundred determinations by taking 1 cc. and making the reaction in a 50 cc. volumetric flask.

**EXPERIMENT 4** Cat 42 (weight, 2353 grams) Three days after the last feeding the animal was anesthetized (ether and morphine), the right kidney was removed, the ureter of the left kidney was ligatured, and 6 grams of creatinine together with about 100 of water were introduced into the ligatured intestine.

	<i>Milligrams</i>
Creatinine nitrogen injected	2172
Creatinine nitrogen absorbed in eighty minutes	822
I Non-protein nitrogen in control blood	31
Urea nitrogen	17

<sup>13</sup> *Journ. of Physiol.* 1910, p. 488.

	<i>Milligrams</i>
II Non-protein nitrogen, carotid artery, nine minutes after the injection	60
Urea nitrogen	18
III Non-protein nitrogen, portal vein, fourteen minutes after the injection	79
Urea nitrogen	18
IV Non-protein nitrogen, portal vein, fifty-one minutes after the injection	91
Urea nitrogen	17
V Non-protein nitrogen, carotid artery, fifty-four minutes after the injection	78
Urea nitrogen	17
VI Non-protein, mesenteric vein, sixty-seven minutes after the injection	104
Urea nitrogen	17
VII Non-protein nitrogen, carotid artery, eighty minutes after the injection	91
Urea nitrogen	18
Non-protein nitrogen in muscle before the injection	216
Urea nitrogen	26
Non-protein nitrogen in muscle eighty minutes after the injection	250
Urea nitrogen	26
Non-protein nitrogen in kidney before the injection	147
Urea nitrogen	19
Non-protein nitrogen in kidney eighty minutes after the injection	181
Urea nitrogen	16

The colorimetric creatinine determinations shattered in this experiment for reasons which we have already described. We had kept some of the samples of blood, however, and later made colorimetric determinations on blood samples IV, V, and VI. The colorimetric values obtained gave us 51, 44 and 70 mgm, respectively, per 100 cc of blood for the creatinine nitrogen. The corresponding direct nitrogen determinations, after subtracting the normal value 31, are 65, 47 and 73.

**EXPERIMENT 5** Cat 44 (weight, 2743 grams) For two weeks prior to the experiment this animal had been kept on a low nitrogen diet consisting of rice (25 grams) and cream (50 cc, 15 per cent fat). For anesthetic we used ether and morphine. Before injecting the creatinine solution the bladder was emptied by means of a syringe.

	<i>Milligrams</i>
Creatinine nitrogen injected	2172
Creatinine nitrogen absorbed in one hundred and twenty-three minutes	922
I Non-protein nitrogen in control blood	30
Urea nitrogen	20
II Non-protein nitrogen, portal vein, one hundred and twenty minutes after the injection	45
Urea nitrogen	21
Creatinine nitrogen, colorimetric determination	12 (15)
III Non-protein nitrogen, mesenteric vein, one hundred and twenty-three minutes after the injection	56
Urea nitrogen	21
Creatinine nitrogen, colorimetric determination	25 (26)
IV Non-protein nitrogen, iliac vein, one hundred and twenty-five minutes after the injection	42
Urea nitrogen	21 (22)
Creatinine nitrogen, colorimetric determination	9
V Non-protein nitrogen, iliac artery, one hundred and twenty-seven minutes after the injection	45
Creatinine nitrogen, colorimetric determination	12 (15)
Non-protein nitrogen in muscle before the injection	130
Urea nitrogen	16
Creatinine nitrogen, colorimetric test	trace
Non-protein nitrogen in muscle one hundred and twenty-seven minutes after the injection	180
Urea nitrogen	16
Creatinine nitrogen, colorimetric determination	54 (50)

The most striking difference in the results recorded in Experiments 4 and 5 is the different distribution of the absorbed creatinine. In the former there is a greater accumulation in the blood, and in the latter the muscles have taken up a greater proportion. It is not possible at the present time to definitely explain this variation. The two experiments are not strictly similar. The absorption periods are longer in Experiment 5, and it is quite likely that the speed of the absorption diminishes as the absorbed product accumulates in the blood, and that the muscles therefore



absorb a larger proportion. Further, Cat 42 had been fed on a protein-rich diet preliminary to the fasting, while Cat 44 (Experiment 5) had been kept on a low nitrogen diet which, especially in a naturally high protein animal like the cat, might leave the muscles unusually low in non-protein nitrogen and thereby lead to a greater avidity for the absorbed products. The non-protein nitrogen per 100 grams of control muscle in Cat 42 is 214 mgm., as against only 130 mgm. in Cat 44. In Cat 42 the elimination of urine had been prevented, but this has little to do with the result, for in the bladder of Cat 44 we obtained only a small quantity of urine secreted during the experiment and it contained only a few milligrams of creatinine. During the last few years numerous interesting and suggestive papers have appeared on creatine and creatinine, on the transformation of the one into the other, and vice versa, as well as on their complete destruction within the body. The results of our absorption experiments seem to throw some new light from a different angle on the problems thus raised. The hypothetical transformation of creatinine into creatine in the muscles, as suggested by Mellanby,<sup>14</sup> or by means of ferments, as claimed by Gottlieb and Stangassinger,<sup>15</sup> is not founded on convincing experiments and is certainly not supported by the determinations recorded above. Considering the nature of our experiments and the somewhat preliminary character of the analytical technique, the colorimetric values obtained for the creatinine in the blood as well as in the muscle correspond surprisingly well with the total increase in the non-protein nitrogen.

To make this point clear we have inserted in brackets opposite the creatinine nitrogen figures the corresponding figures derived from the total non-protein nitrogen determinations.

According to the experiments of Gottlieb and Stangassinger the tissues of the animal body contain not only ferments capable of transforming creatinine into creatine but also ferments capable of decomposing creatine and creatinine. The figures which we have obtained for the urea in the above experiments are however entirely negative so far as the "destruction" of creatinine is concerned. And since our results were obtained from experiments with living animals we are inclined to insist that the results obtained by Gott-

<sup>14</sup> *Journ of Physiol*, **vi**, p 447, 1908

<sup>15</sup> *Zeitschr f physiol Chem*, **lii**, p 1, 1908, **lvii**, p 131, 1908

lieb and Stangassinger in their autolysis and perfusion experiments have no bearing on the processes of animal metabolism. Moreover, the analytical results of Gottlieb and Stangassinger are more or less open to suspicion in view of the circumstance that they did not discover and did not take into account the fact that blood and tissues contain substances which interfere with the colorimetric creatinine determinations. Their conclusion that the figures they obtained represented maximum values because of other substances giving the same reaction is certainly untenable. To be sure Van Hoogenhuyze and Verploegh have verified their results<sup>16</sup> but Melanby on the other hand was unable to do so. At all events our results lend no support to the theory that the animal body contains special ferments for the destruction of creatinine.

#### ABSORPTION OF CREATINE

In the study of the creatine we have made use of the following method

To 10 to 20 cc of the alcoholic blood or tissue extract contained in a large test tube is added 0.1 to 0.2 gram of picric acid dissolved in methyl alcohol and the mixture evaporated to dryness in a boiling water bath. The addition of a few strands of shredded asbestos prevents bumping and facilitates the subsequent solution. When all alcohol has been driven off the tubes are stoppered with tin foil and heated in an autoclave at 130° to 135° for 30 minutes. When cool the creatinine picrate is washed with chloroform, etc., as described above under the determination of creatinine.

EXPERIMENT 6 Cat 43 (weight, 2300 grams) Anesthetic, ether and morphine. For twelve days previous to the experiment this animal had been kept on the low nitrogen rice and cream diet described above. The right kidney was taken out before the injection of 5.7 grams creatine dissolved in about 100 cc of water.

	Milligrams
Creatine nitrogen injected	1595
Creatine nitrogen absorbed in forty-four minutes	387
I Non-protein nitrogen in control blood	31
Urea nitrogen	20
II Non-protein nitrogen, portal vein, fourteen minutes after the injection	35
Urea nitrogen	20

<sup>16</sup> *Zeitschr f physiol Chem*, LVII, p 206, 1908

		<i>Milligrams</i>
III	Non-protein nitrogen, carotid artery, fourteen minutes after the injection	34
	Urea nitrogen	20
	Creatine	trace
IV	Non-protein nitrogen, portal vein, thirty-four minutes after the injection	35
	Urea nitrogen	19
	Creatine nitrogen, colorimetric determination	4
V	Non-protein nitrogen, portal vein, ninety-five minutes after the injection	43
	Urea nitrogen	20
VI	Non-protein nitrogen, mesenteric vein, ninety-six minutes after the injection	50
	Creatine nitrogen, colorimetric determination	17
VII	Non-protein nitrogen, carotid artery, one hundred and two minutes after the injection	41
	Urea nitrogen	20
	Creatine nitrogen, colorimetric determination	12
VIII	Non-protein nitrogen, mesenteric vein, one hundred and four minutes after the injection	77
	Creatine nitrogen, colorimetric determination	48
	Non-protein nitrogen in muscle before the injection	175
	Urea nitrogen	17
	Creatine nitrogen, colorimetric determination	50
	Non-protein nitrogen in muscle one hundred and four minutes after the injection	193
	Urea nitrogen	21
	Creatine nitrogen colorimetric determination	85
	Non-protein nitrogen in right kidney before the injection	140
	Creatine	absent
	Non-protein nitrogen in left kidney one hundred and four minutes after the injection	160
	Creatine nitrogen, colorimetric determination	25

EXPERIMENT 7 Cat 50 (weight, 2293 grams) Anesthetic, ether and morphine This cat had been fed 150 grams of eggs daily for 3 days before the beginning of the experiment but received no food during the last twenty-six hours The results of the preceding experiment showed that it is useless to introduce very much creatine because it precipitates in the intestines In this case we used therefore only 3.6 grams dissolved in about 75 cc of water The kidneys were left undisturbed, but the bladder was emptied before the injection

	Milligrams
Creatine nitrogen injected	1000
Creatine nitrogen absorbed in one hundred and twenty-nine minutes	500
I Non-protein nitrogen in control blood	67
Urea nitrogen	37
II Non-protein nitrogen, jugular vein, forty-five minutes after the injection	70
Urea nitrogen	36
Creatine nitrogen, colorimetric determination	15
III Non-protein nitrogen, carotid artery, one hundred and twenty minutes after the injection	84
Urea nitrogen	36
Creatine nitrogen, colorimetric determination	19
IV Non-protein nitrogen, carotid artery, one hundred and eighty minutes after the injection	94
Urea nitrogen	36
Creatine nitrogen, colorimetric determination	25
V Non-protein nitrogen, iliac vein, one hundred and eighty-three minutes after the injection	94
Urea nitrogen	36
VI Non-protein nitrogen, hepatic vein, one hundred and eighty-nine minutes after the injection	94
Urea nitrogen	38
Creatine nitrogen, colorimetric determination	25
Non-protein nitrogen in muscle before the injection	232
Urea nitrogen	25
Non-protein nitrogen in muscle one hundred and eighty-nine minutes after the injection	270
Urea nitrogen	25

In the last experiment we obtained for the first time a marked absorption of water from the intestine and also a considerable elimination of urine. The urine (30 cc) contained so much creatine that about an hour after we had drawn it, the creatine began to crystallize out. To prevent the crystallization we added 3 drops of concentrated hydrochloric acid and immediately determined the preformed creatinine. The analytical figures (in grams per 100 cc) obtained on the urine are as follows: Total nitrogen, 1.7, urea nitrogen, 0.27, creatinine nitrogen, 0.02, creatine nitrogen, 0.66. This urine also contained 2.65 per cent of sugar. The cat, not a very tame one, got away from us and hid behind the laboratory desks for almost an hour before he was again captured. Notwithstanding the unconsciousness of the animal the "emotional glycosuria," described by Cannon,<sup>1</sup> evidently persisted till the end of the experiment.

<sup>17</sup> *Amer Journ of Physiol*, xxiv, p 280, 1911

With reference to the question of decomposition and urea formation the figures recorded above indicate that none of the absorbed creatine has been converted into urea. The absence of demonstrable decomposition and urea formation is thus in harmony with the "disappearance" of creatine reported by Folin<sup>18</sup> on the basis of feeding experiments with the same substance. Folin's experimental results were more or less identical with those obtained by Klercker and they have since been verified by Van Hoogenhuyze and Verploegh<sup>19</sup> and others. Folin's interpretation with reference to the disappearance of the creatine nitrogen has as yet been neither verified nor disproved, though it has met with considerable skepticism. Van Hoogenhuyze and Verploegh, and Pekelharing<sup>20</sup> as well as Towles and Voegtlin<sup>21</sup> all take for granted that a large part of the creatine which gets into the circulation must be destroyed. The point of view is a perfectly legitimate one as an explanation of the fact that only a very small fraction of injected creatine can be traced to the urine as creatine, and still less, if any, as creatinine. Experimental proof that the nitrogen of injected creatine is eliminated as urea is, however, still conspicuously absent. This is all the more remarkable in view of the chemically unstable character of creatine as compared with the typical urea-formers, the amino-acids. So far as any special destructive or transforming power of the liver is concerned, whether it be of creatine into creatinine or into urea, or of creatinine into creatine or urea, we have failed to find any evidence of it. The blood which contained large quantities of creatine and which must have passed through the liver a great many times gave no stronger creatinine reaction than did the control blood. In this respect our results are therefore in harmony with the recent observations by Towles and Voegtlin<sup>22</sup> on feeding creatine and creatinine to dogs with an Eck's fistula.

#### THE FORMATION OF UREA

In discussing the experimental results obtained with creatine and creatinine we have used the absence of a demonstrable urea for-

<sup>18</sup> Hammarsten's *Festschrift*, 1906

<sup>19</sup> *Loc cit*

<sup>20</sup> *Zeitschr f physiol Chem*, lxxv, p 214, 1911

<sup>21</sup> *This Journal*, x, p 496, 1912

<sup>22</sup> *Loc cit*

mation as an argument against the hypothesis that these two products are extensively destroyed in the body. In doing so we have foreshadowed the point which we propose to prove in this section, namely, the formation of urea from amino-acids under the condition of our absorption experiments.

In our earlier work we were particularly bent on showing and emphasizing the fact and the speed of the absorption of amino-acids, and consequently made experiments of comparatively short duration. The result was a rapid absorption unaccompanied by any unmistakable urea formation. The experiments which we now wish to report were planned specifically for the purpose of showing the formation of urea from the absorbed products, hence we have as a rule drawn no blood during the first half hour after the injection, and have continued the experiments longer than in our earlier work—from two to four hours.

# ALANINE ABSORPTION AND UREA FORMATION

EXPERIMENT 8 Cat 46 (weight, 2030 grams) In the early stages of pregnancy Last feeding (meat) twenty-four hours before the operation Anesthetic, ether and morphine

	<i>Milligrams</i>
Alanine nitrogen injected (in 70 grams of water)	1500
Alanine nitrogen absorbed in one hundred and sixty-eight minutes	600
I Non-protein nitrogen, control blood	41
Urea nitrogen	25
II Non-protein nitrogen, carotid artery, sixty-two minutes after the injection	49
Urea nitrogen	26
III Non-protein nitrogen, carotid artery, one hundred and thirty-two minutes after the injection	56
Urea nitrogen	36
IV Non-protein nitrogen, portal vein, one hundred and sixty-eight minutes after the injection	70
Urea nitrogen	38
Non-protein nitrogen in muscle before the injection	194
Urea nitrogen	26
Non-protein nitrogen in muscle one hundred and eighty minutes after the injection	232
Urea nitrogen	41

EXPERIMENT 9 Cat 53 (weight, 1193 grams) Young animal fed on rice and cream (eight days)

	<i>Milligrams</i>
Alanine nitrogen injected (in 35 grams of water)	500
Alanine nitrogen absorbed in one hundred and eighty-two minutes	458
I Non-protein nitrogen, control blood	35
Urea nitrogen	17
II Non-protein nitrogen, carotid artery, seventy-four minutes after the injection	65
Urea nitrogen	23
III Non-protein nitrogen, carotid artery, one hundred and eighty minutes after the injection	88
Urea nitrogen	35
IV Non-protein nitrogen, hepatic vein, one hundred and eighty-two minutes after the injection	88
Urea nitrogen	35
Non-protein nitrogen in muscle <sup>23</sup> before the injection	216
Urea nitrogen	30
Non-protein nitrogen in muscle <sup>23</sup> one hundred and eighty-two minutes after the injection	248
Urea nitrogen	38

#### GLYCOCOLL ABSORPTION AND UREA FORMATION

EXPERIMENT 10 Cat 47 (weight, 2293 grams) Fed on meat for eight days Last feeding, twenty-eight hours before the operation Anesthetic, ether and chloretone

	<i>Milligrams</i>
Glycocoll nitrogen injected (in 100 grams of water)	1867
Glycocoll nitrogen absorbed in two hundred and thirty minutes	917
I Non-protein nitrogen, control blood	60
Urea nitrogen	34
II Non-protein nitrogen, carotid artery, one hundred and twenty minutes after the injection	100
Urea nitrogen	44
III Non-protein nitrogen, carotid artery, one hundred and eighty minutes after the injection	101
Urea nitrogen	50
IV Non-protein nitrogen, portal vein, two hundred and twenty-two minutes after the injection	128
Urea nitrogen	58

<sup>23</sup> Only 2.5 grams muscle (gracilis) taken for analysis

	<i>Miligrams</i>
V Non-protein nitrogen, iliac artery, two hundred and thirty minutes after the injection	124
Urea nitrogen	60
Non-protein nitrogen in muscle before the injection	248
Urea nitrogen	42
Non-protein nitrogen in muscle two hundred and forty minutes after the injection	304
Urea nitrogen	54

EXPERIMENT 11 Cat 52 (weight, 1043 grams) This was a very young animal and had been fed on rice and cream for seven days, last feeding being twenty-four hours before operation. Anesthetic, ether and morphine

	<i>Miligrams</i>
Glycocoll nitrogen injected (in 30 grams of water)	558
Glycocoll nitrogen absorbed in one hundred and twenty minutes	158
I Non-protein nitrogen, control blood	29
Urea nitrogen	16
II Non-protein nitrogen, carotid artery, sixty minutes after the injection	42
Urea nitrogen	24
III Non-protein nitrogen, carotid artery, one hundred and twenty minutes after the injection	56
Urea nitrogen	30
Non-protein nitrogen in muscle <sup>24</sup> before the injection	200
Urea nitrogen	24
Non-protein nitrogen in muscle <sup>24</sup> one hundred and twenty minutes after the injection	236
Urea nitrogen	30

In addition to the four experiments recorded above on the urea formation from amino-acids, we have made many others, but as they all show substantially the same thing and nothing more, it seems hardly worth while to publish them. Those already cited prove definitely that at the end of an hour or more the formation of urea from the absorbed amino-acids has begun and is unmistakably demonstrable. In fact we have some experiments where the urea increase is marked at the end of 45 minutes. When substances other than pure amino-acids are injected the urea formation begins much earlier. This is true, for example, for Witte's peptone

<sup>24</sup> Only 2.5 grams muscle (*gracilis*) taken for analysis



## PEPTONE ABSORPTION AND UREA FORMATION

EXPERIMENT 12 Cat 51 (weight, 3593 grams) Last feeding (meat) twenty-six hours before the operation Anesthetic, ether and morphine

	<i>Milligrams</i>
Peptone nitrogen injected	500
Peptone nitrogen absorbed in one hundred and ninety-three minutes	225
I Non-protein nitrogen, control blood	54
Urea nitrogen	26
II Non-protein nitrogen, carotid artery, twenty-three minutes after the injection	66
Urea nitrogen	34
III Non-protein nitrogen, carotid artery, one hundred and ten minutes after the injection	72
Urea nitrogen	40
IV Non-protein nitrogen, iliac vein, one hundred and seventy-five minutes after the injection	86
Urea nitrogen	54
V Non-protein nitrogen, iliac artery one hundred and eighty minutes after the injection	86
Urea nitrogen	54
VI Non-protein nitrogen, hepatic vein, one hundred and ninety-three minutes after the injection	85
Urea nitrogen	54
Non-protein nitrogen in muscle before the injection	200
Urea nitrogen	27
Non-protein nitrogen in muscle one hundred and ninety-three minutes after the injection	248
Urea nitrogen	42

The results obtained with such complex mixtures as Witte's peptone are, of course, less clear cut and much more difficult to interpret than are the results obtained with pure amino-acids. Peptone contains not only more or less ammonia but in addition contains amid nitrogen, so that we do not know how far our urea figures represent real deamination. It is interesting to note, however, that even with such a product the "urea" nitrogen obtained from the hepatic blood is not larger than the urea in the blood obtained at about the same time from the iliac artery. The liver has not brought about any demonstrable specialized deamination.

Our experimental data prove that the absorption of amino-acids

is, after a while, accompanied by the formation of urea. They do not furnish any definite evidence as to where the urea is formed. In the absence of satisfactory proof that deamination and urea formation is localized we are not justified in assuming that the process is a specialized process in the sense of being confined to some particular organ. In other words, so far as we yet know, the urea forming process is one characteristic of all the tissues and by far the greatest amount of the urea is therefore probably formed in the muscles. The negative results, so far as any localized urea formation is concerned, is almost satisfactory proof that there is none, for if there were one central focus from which all or nearly all of the urea originated we could scarcely have failed to find it.

The hypothesis that the urea forming process is thus probably largely a matter of muscle metabolism predicates of course nothing as to the nature of the process. In no way does it invalidate or weaken the prevailing view that deamination rather than oxidation represents the first step in the formation of urea from amino-acid nitrogen. Ascribing to the muscles the greatest share in the urea formation represents, therefore, in no essential point a return to the earlier teachings of Pfluger or of Voit.

The important issue between Pfluger and Voit was the highly speculative one as to whether the food protein after absorption had to become living protoplasm before being destroyed, or whether it was decomposed as circulating protein. That problem is, however, now definitely settled by our results. The food protein reaches the tissues in the form of amino-acids and those amino-acids which are not needed for the rebuilding of broken down body material are not rebuilt either into protein or protoplasm, but are broken down and their nitrogen converted into urea. Thus there is virtually nothing left of the older teaching of Pfluger and Voit on this particular subject.

On the other hand new problems are now pressing for answer. What is the starting impulse and the controlling factor in the urea formation? Does a given amount of absorbed nitrogen yield the same amount of urea in a given time whether it is represented by many or by only one kind of amino-acids? In other words does a certain total concentration of amino-acid nitrogen result in urea formation or do the tissues maintain a separate nitrogen equilibrium on the basis of each individual amino-acid? From a teleo-

logical standpoint it would seem almost certain that each tissue would deal individually with each kind of available amino-acids, and would tend to maintain the kind of mixture most suitable to its needs. If such is the case, each tissue maintains a certain supply of each amino-acid and the urea formation from any particular amino-acid depends, so to speak, on the "partial pressure" of that particular acid. This appears to us the simplest tentative hypothesis concerning the urea formation out of the surplus food protein

# ON THE NON-ENZYMATIC CHARACTER OF OOCYTIN (OOCYTASE)

By T. BRAILSFORD ROBERTSON

(From the Rudolph Spreckels Physiological Laboratory of the University of California)

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## 1 INTRODUCTION

In a previous communication I have shown<sup>1</sup> that the active agent in ox-serum, which brings about the formation of fertilization-membranes in sea-urchin eggs, can be isolated by a process consisting, essentially, in precipitation by barium chloride, resolution of this precipitate in dilute acid, and reprecipitation by acetone.

This fertilizing agent, as Loeb has shown<sup>2</sup> and as I have also found, is thermostable, resisting an exposure of nineteen hours to a temperature of 58°C. It is possibly derived from the breaking down of leucocytes in shed blood. Consequently it appears to present some analogies to the *cylases* or cell-liquefying substances shown by Metchnikoff<sup>3</sup> to be derivable from white corpuscles. In the communication to which I have referred I therefore suggested that this substance be termed *oocytase*.

The substances (other than those which occur in sperm, tissue-extracts, etc.) which are known to bring about membrane-formation in sea-urchin eggs are not enzymatic in character. As Loeb has pointed out,<sup>4</sup> the agencies which bring about membrane-formation are haemolytic agents, to wit, besides heat, (1) certain specific substances, such as saponin or bile-salts, (2) a series of fat dis-

<sup>1</sup> T. Brailsford Robertson. *This Journal*, xi, p. 339, 1912.

<sup>2</sup> J. Loeb. *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, p. 187, 1909.

<sup>3</sup> E. Metchnikoff. *L'Immunité dans les maladies infectieuses*, Paris, 1902.

<sup>4</sup> J. Loeb. *loc cit.*, p. 132.

solving agents such as benzene, ether, alcohol and fatty acids, (3) distilled water, (4) hydrogen ions and (5) hydroxyl ions. So far as is known at present no enzymes are capable of bringing about membrane-formation. It therefore appeared of importance to ascertain whether, or not, the fertilizing agent which occurs in tissue-fluids such as blood-serum is an enzyme. Accordingly the following investigations were undertaken.

## 2 EXPERIMENTAL

### *Preparation of the oöcytin (oocytase)*

To 3800 cc of defibrinated and centrifugalized blood were added 590 cc of  $\frac{5M}{2}$  NaCl, making the total volume 4390 cc.<sup>6</sup> To this were added 2190 cc of 7 per cent BaCl<sub>2</sub> solution. A thick cloud appeared in the mixture. Portions of the mixture were heated to from 30° to 40° for about one hour and the remainder (over 4 liters) was kept in a warm room for some hours. The mixture deposited a membranous white precipitate, it was allowed to stand on ice over night. The following day the mixture was centrifuged, the entire precipitate being collected in eight 50 cc centrifuge tubes. The precipitate, after having been thoroughly drained, was stirred up in 500 cc of 2 per cent BaCl<sub>2</sub> solution and again centrifuged and thoroughly drained. This was repeated three times. The precipitate was then stirred up in 200 cc of  $\frac{N}{10}$  HCl rapidly and continuously for one hour, most of the precipitate dissolved, but some residue remained. To this mixture were added 20 cc of 10 per cent Na<sub>2</sub>SO<sub>4</sub> solution, while stirring. The mixture was then allowed to stand at 38°C over night.

The following morning this mixture was centrifuged and the supernatant fluid was filtered. To the clear, white, slightly opalescent filtrate, which was free from barium (yielded no precipitate or cloud on further addition of Na<sub>2</sub>SO<sub>4</sub>), was added 1 liter of acetone (Merck's H. P.). A copious flocculent precipitate formed and settled quickly. This was collected upon a hardened filter, washed twice in 200 cc of alcohol and once with 200 cc of ether and dried over H<sub>2</sub>SO<sub>4</sub> at 38° over night. The following day it was pulverized and sifted and obtained in the form of a perfectly white friable powder. The total weight of the product obtained was 1.06 grams. About one-eighth of the material was lost through the breaking of a centrifuge-tube, so that the total amount which might have been obtained from the volume of serum employed was 1.2 grams, corresponding to a concentration of 0.03 per cent in the serum.

The substance was spread out in a thin layer to dry over H<sub>2</sub>SO<sub>4</sub> at 38° for some days. Careful flame tests, both before and after moistening the powder with nitric acid, failed to reveal the presence of any trace of barium.

<sup>6</sup> I have observed that if excess of NaCl is added at this point precipitation by barium is greatly delayed.

The substance thus obtained was an exceedingly potent fertilizing and cytolyzing agent. At a dilution of one to half a million it caused membrane-formation in 100 per cent of sensitized<sup>6</sup> eggs of *Strongylocentrotus purpuratus* within ninety minutes. At a dilution of one to twenty-five thousand it caused marked cytolysis in a like period of time.

### Comparison with trypsin (alkali-proteinnase)

I prepared a 2 per cent solution of "basic" potassium caseinate,<sup>7</sup> neutral to phenolphthalein, by dissolving 8 grams of casein in 64 cc of  $\frac{N}{10}$  KOH which had previously been diluted to 400 cc. The solution was filtered before using in order to remove a very slight trace of undissolved residue.

Three 100 cc samples of this solution were then taken and separately placed in tightly-stoppered Erlenmeyer flasks of 200 cc capacity. From two to three drops of toluol were added to each sample and they were then warmed to 35°C.

Three cubic centimeters of a solution containing 50 mg of oocytin were prepared by dissolving 50 mg of oocytin in 1.5 cc of  $\frac{N}{10}$  HCl and then accurately neutralizing this solution by the addition of 1.5 cc of  $\frac{N}{10}$  KOH.

A 0.4 per cent solution of trypsin was prepared by dissolving Grüber's trypsin puriss. sicc. in distilled water.

The 100 cc samples of 2 per cent potassium caseinate were then treated as follows:

To number 1 added 3 cc of distilled water.

To number 2 added 3 cc of 0.4 per cent trypsin solution (= 12 mg trypsin).

To number 3 added 3 cc of solution containing 50 mg oocytin.

The mixtures thus prepared were then returned to the incubator at 35°C.

From time to time, as detailed below, 10 cc samples were taken from each flask and the quantity of casein hydrolyzed in each mixture was determined by a method previously described.<sup>8</sup> The following were the results obtained:

TIME OF DIGESTION	GRAMS CASEIN PER 100 CC HYDROLYZED		
	Mixture containing no trypsin or oocytin	Mixture containing 12 mg trypsin per 100 cc	Mixture containing 50 mg oocytin per 100 cc
1 hour	0.0	0.3	0.0
3 hours	0.0	0.9	0.0
6 hours	0.0	1.8	0.0
23 hours	0.0	2.1	0.0

<sup>6</sup> By four minutes' immersion in  $\frac{3M}{8}$  SrCl<sub>2</sub>.

<sup>7</sup> For the methods employed in purifying the casein, preparing solutions of the caseinates of known reaction, etc., cf T Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden, 1912.

<sup>8</sup> T Brailsford Robertson *this Journal*, xii, p. 23.

It is clear, therefore, that in faintly alkaline solutions oocytin exerts no measurable proteolytic action, even when present in a concentration four times as great as the concentration of trypsin which brings about complete hydrolysis of the casein in 2 per cent solution after twenty-three hours. We may conclude that oocytin possesses no detectible tryptic activity, *it is not an alkali-proteinase*

#### *Comparison with pepsin (acid-proteinase)*

If pepsin (acid-proteinase) be allowed to act upon casein in neutral or faintly acid solution, after the lapse of some time a considerable quantity of a phosphorus-rich substance (paranuclein), which is protein in nature and insoluble in such solutions, is deposited.<sup>9</sup> This phenomenon may be utilized as an indication of the presence or absence of acid-proteinase in a neutral or faintly acid mixture containing casein.

A 4 per cent solution of "neutral" potassium caseinate, neutral to litmus, was prepared by dissolving 4 grams of casein in 20 cc of  $\frac{N}{10}$  KOH which had been previously diluted to 100 cc. This solution was filtered and obtained perfectly clear and free from any cloudiness or other indication of suspended matter. Ten cubic centimeter samples of this solution were placed in tightly-stoppered test-tubes and warmed to 35°C after the addition of two drops of toluol to each of them.

Fifty milligrams of oocytin were dissolved in 5 cc of  $\frac{N}{10}$  HCl and this solution was accurately neutralized by the addition of 5 cc of  $\frac{N}{10}$  KOH. After warming this solution to 35°C it was added to one of the 10 cc samples of casein solution and the mixture was set aside at 35°C.

Fifty milligrams of Grubler's pepsin puriss sicc were dissolved in 10 cc of distilled water and, after warming to 35°C, this solution was added to another of the 10 cc samples of casein solution, and this mixture was also set aside at 35°C.

After the lapse of a week the two mixtures presented a marked contrast. The mixture containing pepsin was milky and turbid and had deposited a bulky precipitate of paranuclein. The mixture containing oocytin was perfectly clear and had deposited no precipitate.

We may conclude, therefore, that in comparison with Grubler's pepsin, oocytin exerts no demonstrable proteolytic action in neutral or faintly acid solutions, *it is not an acid-proteinase*.

<sup>9</sup> Cf. Lubavin *Hoppe-Seyler's Med Chem Untersuch*, Berlin, 1866, p 463, A. Kossel *Verhandl d berl physiol Gesellsch*, *Arch f (Anat u) Physiol*, 1891, p 181, T Brailsford Robertson *this Journal*, III p 95, 1907, v, p 493, 1909.

*Comparison with lipase*

A 1 per cent solution of Kahlbaum's triacetin was prepared and divided into two portions of 100 cc each which were warmed to 32°C. To the one portion was then added a mixture of 5 cc of  $\frac{N}{10}$  HCl plus 5 cc of  $\frac{N}{10}$  KOH and to the other a solution of oöcytin prepared by dissolving 50 mg of oöcytin in 5 cc of  $\frac{N}{10}$  HCl and neutralizing with 5 cc of  $\frac{N}{10}$  KOH. The mixtures thus prepared were returned to the incubator at 32°C.

After four hours one drop of 2 per cent alcoholic phenolphthalein was added to a 10 cc sample of each mixture, and these samples, after dilution with water, were titrated against  $\frac{N}{10}$  KOH. One drop of the  $\frac{N}{10}$  KOH turned each sample red, consequently no appreciable splitting of the triacetin had occurred in four hours.

To each of the mixtures were now added three drops of toluol and they were returned to the incubator. After twenty-four hours 10 cc samples were again tested in the manner described above and with a like result.

A 10 cc sample of either mixture, after the triacetin had been completely saponified by successive additions of  $\frac{N}{10}$  KOH and boiling, neutralized 12.5 cc of  $\frac{N}{10}$  KOH.

We may therefore conclude that oöcytin does not hydrolyze triacetin in neutral solutions.

An impure lipase-powder was prepared from ripe castor (*Ricinus*) beans in the following manner. The shells were removed by hand without the use of water. The seeds were then macerated and extracted with alcohol for a day, drained and then washed with ether and dried by spreading out the powder in a thin layer in a warm room.<sup>10</sup>

Two cubic centimeters of Kahlbaum's triolein were delivered into each of four 200 cc Erlenmeyer flasks labelled, respectively, A, B, C and D.

To A were added 100 cc of distilled water.

To B were added 100 cc of water containing 50 mg of oöcytin dissolved in 1 cc of  $\frac{N}{10}$  HCl, this solution having been neutralized by the addition of 1 cc of  $\frac{N}{10}$  KOH.

To C were added 200 mg of lipase-powder rubbed up in 100 cc of water containing 1 cc of  $\frac{N}{10}$  HCl + 1 cc  $\frac{N}{10}$  KOH.

To D were added 200 mg of lipase-powder rubbed up in 100 cc of water containing 50 mg of oöcytin dissolved in 1 cc of  $\frac{N}{10}$  HCl and neutralized by the addition of 1 cc of  $\frac{N}{10}$  KOH.

The flasks were then tightly stoppered and shaken rapidly in a shaking machine which was placed in a room heated to 32°C and maintained at a constant temperature. After ten minutes' shaking 10 cc samples of each mixture were taken, 50 cc of absolute alcohol were added to each sample, and they were titrated against phenolphthalein with  $\frac{N}{10}$  NaOH in alcoholic

<sup>10</sup> Cf A. E. Taylor, *On Fermentation*, Univ Calif Publ Pathol, 1, p 258, 1907.



solution <sup>11</sup> This procedure was repeated from time to time with the results shown in the following table

*Cubic centimeters of  $\frac{N}{10}$  NaOH required to neutralize 10 cc of the digestion mixture*

	A	B	C	D
After 10 minutes	0 3	0 3	0 4	0 4
After 24 hours	0 35	0 35	0 65	0 6
After 48 hours	0 5	0 4	2 0	1 9

It is evident that the hydrolysis due to the Ricinus lipase took place more rapidly in the second twenty-four hours than in the first, a phenomenon which has been observed by Hoyer<sup>12</sup> and attributed by him to the setting free of auto-catalysors during the progress of the hydrolysis

It is further evident, *firstly* that oocytin does not accelerate the hydrolysis of triolein in neutral solution, and *secondly* that it does not aid the hydrolysis of triolein by Ricinus lipase From these experiments and from the experiments detailed above, in which triacetin was employed as substrate, we may conclude that in all probability *oocytin is not a lipase, nor does it act as a co-ferment for Ricinus lipase*

#### *Comparison with emulsin*

A 1 per cent solution of amygdalin (Merck, H P ), was divided into two portions of 100 cc each To the one portion I added 5 cc of  $\frac{N}{10}$  HCl and 5 cc of  $\frac{N}{10}$  KOH which had been previously mixed, to the other 50 mg of oocytin dissolved in 5 cc of  $\frac{N}{10}$  HCl and neutralized by the addition of 5 cc of  $\frac{N}{10}$  KOH To 10 cc samples of each of these mixtures were then added 10 cc each of Fehling's solution 1 and 2 prepared according to the formula of Bertrand,<sup>13</sup> they were then boiled for three minutes No precipitate or cloud was produced, consequently the mixtures were free from glucose

The mixtures were then placed in an incubator at 32°C and tested from time to time with Fehling's solution After twenty-four hours these tests failed to indicate the presence within the mixtures of even a trace of glucose In a like period at 32° I have found that 50 mg of Merck's emulsin will bring complete hydrolysis of the amygdalin in 100 cc of a 1 per cent solution with the liberation of nearly 60 mg of glucose per 10 cc

<sup>11</sup> Cf H C Bradley this *Journal*, viii, p 251, 1910

<sup>12</sup> E Hoyer *Zeitschr f physiol Chem*, i, p 414, 1907

<sup>13</sup> Bertrand *Bull soc chim*, xxxv, p 1285, 1906

We may therefore conclude that *oöcytin* does not accelerate the hydrolysis of  $\beta$ -glucosides<sup>14</sup>

### *Comparison with peroxidase*

To a few cubic centimeters of a 0.05 per cent solution of oöcytin (in  $\frac{N}{20}$  KCl, prepared by dissolving the oöcytin in  $\frac{N}{10}$  HCl and neutralizing with  $\frac{N}{10}$  KOH) I added a drop of tincture of guaiacum. No coloration was observed. I then added several drops of hydrogen peroxide solution (3 per cent), still no coloration was observed. One drop of defibrinated ox-blood diluted to several cubic centimeters and treated with hydrogen peroxide and guaiacum in the proportions indicated yielded a deep blue coloration at once.

We may conclude, therefore, that *oöcytin* is not a peroxidase.

So far as the above observations go, therefore, although far from complete, it will be seen that they afford no support for the view that the membrane-forming and cytolyzing action of oöcytin is attributable to enzymatic activity. Having regard to the fact that the chemical agents (haemolytic agents) which are known to cause membrane-formation are in no case enzymes, we may, I think, conclude that the membrane-forming agent which may be isolated from mammalian blood-serum by the method outlined above is not an enzyme. This being the case, the name *oöcylase*, which I originally suggested should be applied to this substance, becomes inappropriate, since the termination *ase* connotes an enzyme. I now propose that the membrane-forming agent in blood-sera be termed *oöcytin*.

### 3 THE NON-ENZYMATIC CHARACTER OF THE FERTILIZING AGENT IN SPERMATOOZOA

In a previous communication<sup>15</sup> I have shown that a membrane-forming agent can be extracted from spermatozoa which resembles oöcytin in being thermostable, precipitable by salts of the alkaline earths and by acetone, and soluble in dilute acids. So far as our knowledge extends at present the membrane-forming agent in echinoderm spermatozoa is indistinguishable from the membrane-forming agent in mammalian blood-sera, and I have therefore

<sup>14</sup> Cf. E. Frankland Armstrong, *The Simple Carbohydrates and the Glucosides*, London, 1910, p. 55.

<sup>15</sup> T. Brailsford Robertson, *this Journal*, xii, p. 1, 1912.

advanced the hypothesis that they are identical. If this be so then it would appear probable that the membrane-forming agent in spermatozoa is not an enzyme.

I have been able to confirm this deduction experimentally in the following manner. It has been shown by Loeb<sup>16</sup> that the fertilization-membrane is formed by the dissolution of a lipid emulsion at the surface of the egg, followed by the osmotic imbibition of water. It has further been observed by v. Knaffl-Lenz and by Loeb that if sea-urchin eggs in which membrane-formation has occurred be placed in sea-water to which blood-serum, white of egg or tannic acid has been added, the membrane collapses. Loeb interprets this to mean that the membrane is impermeable to colloids. The membrane can be shown to be permeable for salts,<sup>17</sup> accordingly the presence of the colloid in the outer sea-water creates an osmotic tension which results in the withdrawal of fluids from the membrane and its subsequent collapse.

I have amplified these experiments<sup>18</sup> and have shown that it is possible to delay or even altogether inhibit the membrane-forming action of a variety of agents (butyric acid, blood-serum, sperm, saponin) by the addition of proteins (such as ovomucoid, casein, serum proteins or gelatin) to the outer medium bathing the eggs. I have furthermore shown that the order of effectiveness of various proteins in inhibiting membrane-formation is the reverse order of their ability to pass through a porcelain filter, thus confirming the views of Loeb, cited above, regarding the mode of formation of the fertilization-membrane.

I have found that the concentration of a given protein which is required to inhibit membrane-formation in sea-urchin eggs varies in a very definite manner with the potency or concentration of the membrane-forming agent. This fact is very strikingly illustrated by the influence of proteins upon membrane-formation by saponin. Thus one part of saponin in 1333 of sea-water causes the formation of perfect spherical membranes in 100 per cent of *purpuratus* eggs within six minutes. The addition of 2 per cent of ovomucoid to the sea-water markedly delays membrane-formation, nevertheless within fourteen minutes 100 per cent of perfect spherical membranes

<sup>16</sup> J. Loeb *loc cit*, p. 125.

<sup>17</sup> J. Loeb *loc cit*, p. 127.

<sup>18</sup> T. Brailsford Robertson *Univ. Calif. Pub. Physiol.*, 14, p. 79, 1912.

are formed. One part of saponin in 64,000 of sea-water causes the formation of perfect spherical membranes in 100 per cent of eggs within thirty-five minutes, but in the presence of 2 per cent ovomucoid only 10 per cent are produced in an hour.

The power of a given concentration of protein in the external medium to inhibit membrane-formation is thus seen to depend upon the quantity of the membrane-forming agent which has penetrated the cell. If a sufficient quantity of the membrane-forming agent enters the cell, then the processes underlying membrane-formation will proceed until a sufficient quantity of water-attracting substance has been set free within the cell to overcome the osmotic tension of a given quantity of protein in the external medium, but if an insufficient quantity of the membrane-forming agent has entered the cell, the processes underlying membrane-formation may cease before this point is reached.

Now *purpuratus* eggs which have been "sensitized" by four minutes' immersion in  $\frac{3}{4}$  M  $\text{SrCl}_2$  will usually form perfect spherical fertilization-membranes when immersed in undiluted isotonic ox-serum.<sup>19</sup> But *purpuratus* spermatozoa are unable to cause membrane-formation in eggs immersed in such serum. Moreover if eggs are previously fertilized by sperm and then immersed in such serum, their membranes collapse, as already stated, and this collapse of the membranes occurs even if the eggs are transferred to the serum over half an hour after fertilization.

From these facts it would appear, *first*, that the "charge" of membrane-forming material which a *purpuratus* egg gains from the entry of a single sperm is not so great as that which it can accumulate, after sensitization, when immersed in a potent undiluted serum, and *secondly* that the membrane-forming agent in spermatozoa is not an enzyme, for if it were and its membrane-forming action were due to its enzymatic activity, then in the course of time the amount of change (or of water-attracting material) produced by a small "charge" of the membrane-forming agent should be the same as that produced by a larger "charge," just as the amount of digestion brought about by 1 mg of trypsin is ultimately the same, or nearly the same, as that which is brought about by 2 mg of trypsin, although it takes place at one-half the velocity.

<sup>19</sup> Prepared by adding 10 cc of  $\frac{5}{8}$  M NaCl to 65 cc of ox-serum.

In other words, were the membrane-forming action of spermatozoa due to the action of an enzyme, then in the course of time, if not immediately the membrane appears, the amount of water-attracting material set free in the egg should be sufficient to overcome the osmotic resistance offered by any concentration of protein which will permit membrane-formation at all

It appears rather probable, therefore, that the membrane-forming agent in echinoderm spermatozoa, like that in mammalian blood-sera, is not an enzyme

#### 4 PROPERTIES OF OOCYTIN

The following properties of oocytin have been determined or confirmed since the appearance of my original communication and may be briefly referred to here pending more extended and detailed investigation

The substance, when pure, is perfectly white and yields, when dissolved in dilute acids, colorless, markedly opalescent solutions which, upon neutralization, become even more opalescent. From its general behavior the substance would appear to be a colloid. I have been unable to coagulate it by heating.

In response to various color-tests it yields the following results

*Millon's test* an atypical very faint reddish color which disappears with prolonged boiling

*Xanthoproteic test* negative

*Biuret test* negative

*Acree's formaldehyde test* very faintly but unmistakably positive

From these results it would appear that oöcytin is not a protein, the faint positive reaction obtained with Acree's test being probably due to slight contamination with adherent protein

*Molisch's test* is also negative

*Pettenkofer's test* is negative

No precipitate or increase in opalescence is produced in a 0.2 per cent solution of oocytin by two-thirds saturation with ammonium sulphate

On mixing 3 cc of a solution of oocytin containing 50 mg with 100 cc of a 1 per cent solution of amygdalin a very pronounced cloud is produced in the solution, which, however, does not settle out

Oocytin, even in minute concentrations (1 part in 2000), exerts a very interesting action upon mixtures of triolein and water. If 2 cc of triolein<sup>20</sup> be shaken violently with 100 cc of distilled water for from twenty-four to forty-eight hours a milky emulsion is formed, having a very thin layer of oil floating upon the top. If the distilled water contains 1 part in 2000 of oocytin, however, no such emulsion is formed, and discrete drops of oil float to the top immediately the shaking is discontinued, leaving the supernatant fluid clear and transparent. It is perhaps not without significance, in view of the membrane-forming potency of the fatty acids, that  $\frac{N}{10}$  acetic acid has a similar action. Our knowledge of the mechanics of emulsion-formation is, at present, however, too incomplete to permit a safe interpretation of these facts.

## 5 CONCLUSIONS

1 Oocytin, the membrane-forming agent in mammalian blood-sera (formerly termed oocytase), is neither an alkali-proteinase, an acid-proteinase, a lipase nor a peroxidase. It does not accelerate the hydrolysis of  $\beta$ -glucosides. It does not act as a co-ferment for Ricinus lipase.

2 It is inferred that oocytin is probably not an enzyme.

3 The membrane-forming agent in *Strongylocentrotus purpuratus* spermatozoa is also probably not an enzyme.

4 Oocytin is not a protein.

<sup>20</sup> Vide the experiments enumerated under *Comparison with Lipase*, part 2 of this article.



# HYDANTOINS THE SYNTHESIS OF THIOTYROSINE<sup>1</sup>

## TWELFTH PAPER

By TREAT B JOHNSON AND CHARLES A BRAUTLECHT

(From the Sheffield Laboratory of Yale University)

(Received for publication June 4, 1912)

In a preliminary paper<sup>2</sup> from this laboratory, entitled "Sulphur in Proteins," the writer wrote as follows "If now sulphur proteins be viewed as oxygen proteins in which bivalent sulphur has partially displaced oxygen, one therefore might expect to find two types of sulphur linkings in sulphur proteins corresponding to the two important primary oxygen groupings, viz mercapto,  $-\text{SH}$ , and thioamide,  $-\text{CSNH}-$  In fact, cysteine represents a sulphur compound of the first type, in which such a displacement has taken place in the case of hydroxyl oxygen in serine Thiotyrosine  $\text{HS } \text{C}_6\text{H}_4\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$  has not yet been synthesized" It is now gratifying to write that our expectation has been realized by other workers,<sup>3</sup> since the publication of our paper, and that it has been shown that the thioamide group,  $-\text{CSNH}-$ , actually functions in the synthesis of proteins

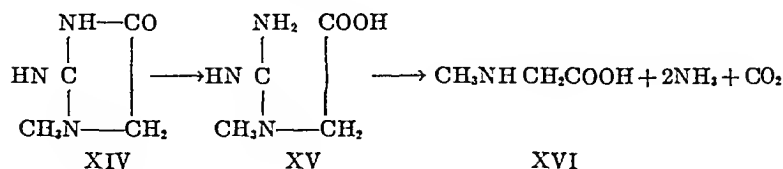
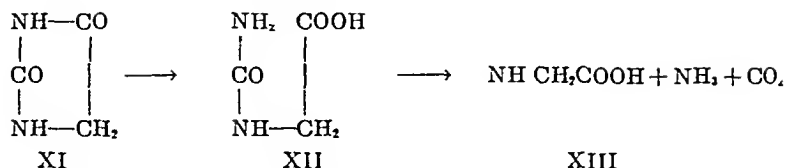
No evidence has yet been produced, so far as the writer is aware, which indicates that an aromatic mercapto grouping,  $-\text{C}_6\text{H}_4\text{SH}$ , is present in sulphur proteins If, however, such a linking is to be considered the most probable cleavage product to look for would be the sulphur analogue of tyrosine, viz *thiotyrosine* Whether this amino-acid will ever be discovered among the products of hydrolysis or not, nevertheless it cannot be excluded from consideration until all of the sulphur in proteins can be accounted for Our

<sup>1</sup> The work described in this paper formed part of a thesis presented by Mr Charles Andrew Brautlecht to the Graduate School of Yale University for the degree of Doctor of Philosophy (T B J)

<sup>2</sup> Johnson and Burnham this *Journal*, ix, p 331, 1911

<sup>3</sup> Barger and Ewin *Journ of Chem Soc* (London), 1911, p 2336





It has also been known for many years that certain organic compounds containing the peptide grouping,  $-\text{NHCH}_2\text{CO}-$ , are capable of condensing with aldehydes,<sup>10</sup> but it was not until recently, however, that it was shown that the methylene group in hydantoin is likewise reactive towards aldehydes and that this cycle condenses smoothly with these reagents forming stable condensation products. This observation was made in this laboratory.<sup>11</sup> These condensation products have proved valuable for synthetic purposes because it is possible to reduce them to substituted hydantoins, which can then be hydrolyzed to amino-acids. In order to obtain thiotyrosine, we employed this new method and synthesized first the hydantoin and phenylhydantoin of this amino-acid.

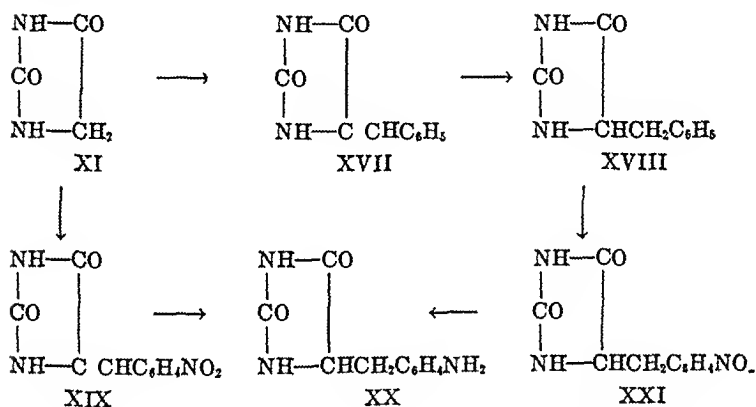
In order to obtain the hydantoin of thiotyrosine we prepared first the hitherto unknown hydantoin of para-aminophenylalanine. This was accomplished as follows. Benzalhydantoin (XVII) was prepared by condensation of hydantoin (XI) with benzaldehyde<sup>12</sup> and converted into benzylhydantoin (XVIII) by reduction with sodium amalgam or hydriodic acid. This hydantoin was then subjected to nitration with cold fuming nitric acid.

<sup>10</sup> Plöchl *Ber d deutsch chem Gesellsch*, **vi**, p 2815, Plöchl and Wolfrum *ibid*, **xviii**, p 1183, Hoffman *ibid*, **xix**, p 2554, Erlenmeyer *Ann d Chem* (Liebig), **cclxxi**, p 137, **cclxxv**, p 1, Erlenmeyer and (Bade, Mattes, Stadlin and Wittenberg) *ibid*, **cccxxvii**, pp 205, 222, 265, 271, 283 and 294, *Ber d deutsch chem Gesellsch*, **xxv**, p 3445, Erlenmeyer and Früstuck *Ann d Chem* (Liebig), **cclxxiv**, p 36, **cccvii**, p 73.

<sup>11</sup> *Amer Chem Journ*, **xlv**, p 368, 1911.

<sup>12</sup> Wheeler and Hoffman *Amer Chem Journ*, **xlv**, p 368.

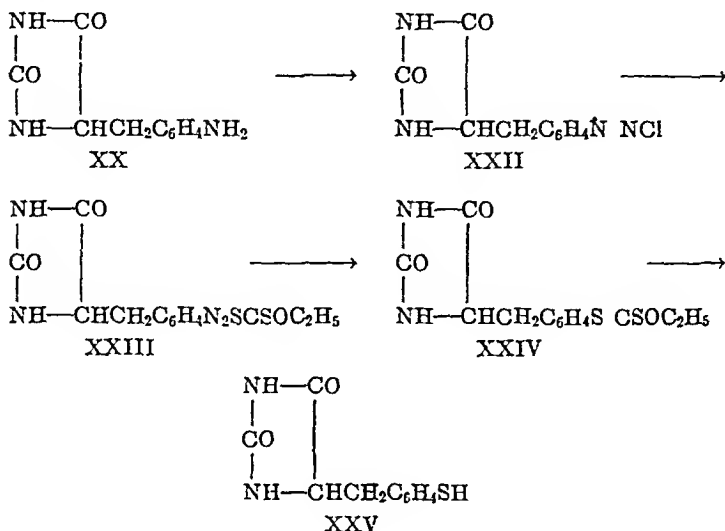
when the corresponding paranitrobenzylhydantoin (XXI) was formed smoothly. The nitrohydantoin was then converted into the corresponding para-aminobenzylhydantoin (XX) by reduction with hydriodic acid. The constitution of this aminobenzylhydantoin and also that of the corresponding nitro compound (XXI) was established by the fact that the same para-aminobenzylhydantoin (XX) was formed by reduction of para-nitrobenzalhydantoin<sup>13</sup> (XIX) with hydriodic acid. These various transformations are represented by the following structural formulas



Thiotyrosine hydantoin (XXV) was prepared from para-aminobenzylhydantoin (XX) in the following manner. This hydantoin was first diazotized in the customary manner and the resulting diazonium salt (XXII) then combined with potassium xanthogenate, in aqueous solution, when the corresponding diazonium-xanthogenate (XXIII) was formed. The yield was nearly quantitative. This salt, however, is unstable and when allowed to stand at ordinary temperature or when warmed in water it undergoes decomposition with evolution of the diazo nitrogen and the xanthogenic ester (XXIV) is formed. This ester was obtained in a crystalline condition and could be purified by crystallization from alcohol. In order to obtain the hydantoin of thiotyrosine this xanthogenate was digested with water when it underwent

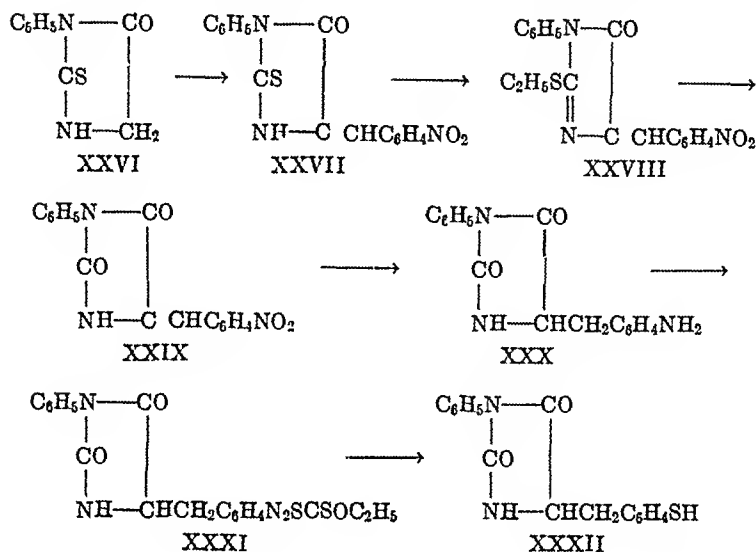
<sup>13</sup> Wheeler and Hoffman *loc cit*

hydrolysis and a quantitative yield of the hydantoin (XXV) was obtained. These changes are represented by the following formulas

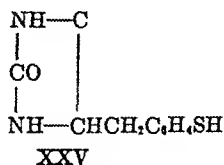
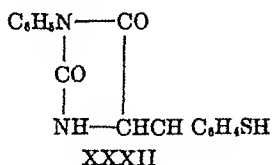


The corresponding 1-phenyl derivative of thiotyrosinehydantoin (XXXII) was synthesized as follows. 1-Phenyl-2-thio-4-paranitrobenzalhydantoin<sup>14</sup> (XXVII) was first converted into its ethylmercapto derivative (XXVIII) by alkylation with ethyl bromide and this compound then digested with hydrochloric acid when the hydantoin (XXIX) was formed with evolution of ethylmercaptan. This compound was then reduced with hydriodic acid and red phosphorus when a quantitative yield of 1-phenyl-4-para-aminobenzylhydantoin (XXX) was obtained. This aminohydantoin was then converted into the diazonium compound and the latter, by the action of potassium vanthogenate, transformed into the diazoxanthogenate (XXXI). The latter (XXXI) gave on hydrolysis the phenylhydantoin of thiotyrosine (XXXII). The various transformations in this synthesis are represented by the following formulas

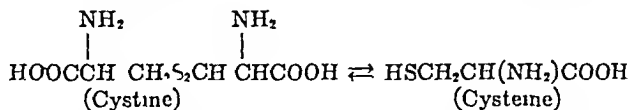
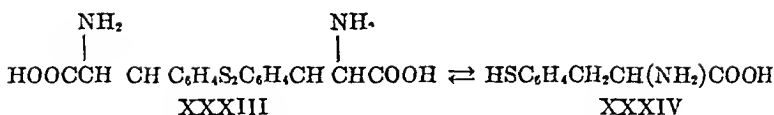
<sup>14</sup> Wheeler and Brautlecht *Amer Chem Journ*, xlv, p 446



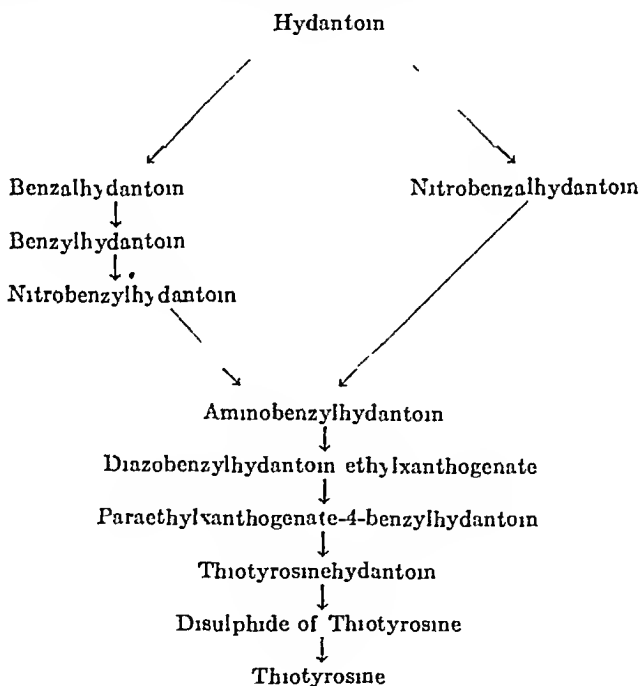
The hydantoin (XXV) and phenylhydantoin (XXXII) of thiotyrosine are stable substances and apparently are not oxidized by the air to their corresponding disulphides. They are both decomposed by heating with a strong, aqueous solution of barium hydroxide with evolution of ammonia and aniline and formation of thiotyrosine (XXXIV). This amino-acid, however, is less stable than its corresponding hydantoin derivatives and undergoes an immediate oxidation in this alkaline solution forming the corresponding disulphide (XXXIII). The latter compound is a well crystallized substance and is very insoluble in water. It is converted quantitatively into thiotyrosine (XXXIV) by reduction with tin and hydrochloric acid. The properties of these two new amino-acids and several of their salts are given in the experimental part of this paper.



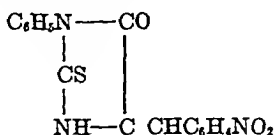
## Synthesis of Thiotyrosine



The following shows the different steps by which the synthesis of thiotyrosine from hydantoin has been effected



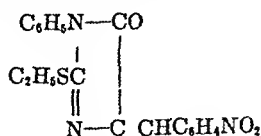
## EXPERIMENTAL PART

*1-Phenyl-2-thio-4-paranitrobenzalhydantoin*

This hydantoin<sup>15</sup> was prepared by condensation of nitrobenzaldehyde with phenylthiohydantoin. The latter was prepared by the action of phenylisothiocyanate on ethyl amino-acetate<sup>16</sup>. The phenylthiohydantoin was condensed with a molecular proportion of nitrobenzaldehyde by digestion for about one hour in 15 parts of glacial acetic acid in the presence of 2 parts of anhydrous sodium acetate. The acid mixture was then poured into a large volume of cold water when most of the condensation product separated. This was separated by filtration and the filtrate evaporated to dryness to remove acetic acid. The sodium acetate was then dissolved with water when the remainder of the condensation product was obtained. After washing the crude hydantoin with cold alcohol and ether it was then purified by recrystallization from boiling glacial acetic acid. It separated on cooling in the form of yellow prisms, which melted at 278° to 279°. The yield was nearly quantitative. The hydantoin is extremely insoluble in the common organic solvents and dissolves in sodium hydroxide solution forming a characteristic sodium salt, which is difficultly soluble in water. The hydantoin is precipitated unaltered by addition of acids to aqueous solutions of its sodium salt.

ANALYSIS (Kjeldahl)	Calculated for $C_{15}H_{17}O_2N_3S$	Found
N	12.92	12.90

*1-Phenyl-2-ethylmercapto-4-paranitrobenzalhydantoin*



Paranitrobenzalhydantoin was first converted into its sodium salt by warming with an alcoholic solution of sodium ethylate, containing one molecular proportion of sodium. Somewhat more than one molecular proportion of ethylbromide was then added and the mixture heated on the steam bath until the reaction was com-

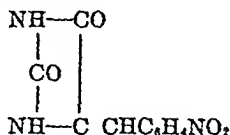
<sup>15</sup> Wheeler and Brautlecht *loc cit*

<sup>16</sup> Marckwald, Neumark and Stelzner *Ber d deutsch chem Gesellsch*, xxiv, p 3278

according to the rate of heating. It was dried for analysis in a desiccator over concentrated sulphuric acid.

ANALYSIS (Kjeldahl)	Calculated for $C_{11}H_{11}O_2N_2 \cdot HNO_3$	Found
N	16.28	16.05

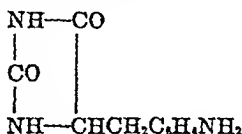
*4-Paranitrobenzalhydantoin*



This compound has been described in a previous paper from this laboratory and was prepared by condensation of hydantoin with parnitrobenzaldehyde.<sup>17</sup> We prepared it according to this method and purified it by crystallization from glacial acetic acid. It melts at 254°.

ANALYSIS (Kjeldahl)	Calculated for $C_{12}H_{11}O_4N_3$	Found
N	18.03	17.91

*4-Para-aminobenzylhydantoin*



This hydantoin was obtained in the form of its hydrochloric acid salt by reduction of the preceding nitro compound with tin and hydrochloric acid. The solvent used was 95 per cent alcohol. After the reduction was complete the solution was then evaporated to dryness to remove the alcohol and hydrochloric acid. The double tin salt was then dissolved in water, the tin precipitated as the sulphide and the solution then evaporated again when the hydrochloride of the hydantoin was obtained. This salt is extremely soluble in water and was purified by crystallization.

<sup>17</sup> Wheeler and Hoffman *loc cit*

from alcohol, which was saturated with hydrochloric acid gas, or from dilute hydrochloric acid. It separated in prisms, which melted with decomposition at  $255^{\circ}$  to  $257^{\circ}$ . The yield was excellent. It was dried for analysis in a desiccator over sulphuric acid.

ANALYSIS	Calculated for $C_{10}H_{11}O_2N_3 \cdot HCl$		Found		
		I	II	III	
N	17.39	17.34	17.44		
Cl	14.70			14.58	

This same hydrochloride can also be obtained by digesting the hydriodide (see below) in aqueous solution with silver chloride. In order to obtain the hydantoin the hydrochloride was dissolved in water and an equivalent quantity of sodium hydroxide added to the solution. The hydantoin separated at once in the form of prisms and was purified for analysis by crystallization from 95 per cent alcohol. It melted at  $145^{\circ}$ .

ANALYSIS (Kjeldahl)	Calculated for $C_{10}H_{11}O_2N_3$	Found
N	20.49	20.57

*Hydriodide of 4-Para-aminobenzylhydantoin*,  $C_{10}H_{11}O_2N_3 \cdot HI$ . This salt is formed quantitatively by reduction of paranitrobenzylhydantoin with hydriodic acid and phosphorus. After removing the red phosphorus by filtration and the excess of hydriodic acid by evaporation the crystalline hydriodide was obtained. It was purified by crystallization from 95 per cent alcohol and separated, on cooling, in needles which decomposed at  $220^{\circ}$ . It was dried for analysis in a desiccator over sulphuric acid.

ANALYSIS	Calculated for $C_{10}H_{11}O_2N_3 \cdot HI$		Found	
		I	II	III
N	12.61	2.39		
I	38.14		37.77	37.99

#### *The Formation of Tyrosinehydantoin from 4-Para-aminobenzylhydantoin*

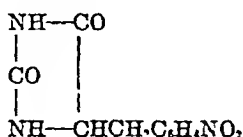
One gram of the above para-amino derivative was diazotized in the usual manner, and the corresponding diazonium salt then decomposed by heating on the steam bath. Nitrogen was evolved



and a crystalline substance was obtained, which was identified as tyrosinehydantoin. The yield was 0.8 gram and it melted at  $258^{\circ}$ . When mixed with some pure tyrosinehydantoin, prepared from anisalhydantoin by reduction with hydriodic acid, this melting point was not altered.

ANALYSIS (Kjeldahl)	Calculated for $C_{10}H_{12}O_3N_2$	Found	
		I	II
N	13.59	13.32	13.46

*4-Paranitrobenzylhydantoin*



The hydantoin of phenylalanine, which was used in this experiment, was prepared by reduction of benzalhydantoin<sup>18</sup> with sodium amalgam. The nitro group was introduced by nitration at  $0^{\circ}$  with nitric acid of specific gravity 1.52. For one part of the hydantoin we used 3 parts of the nitric acid. After nitration, the acid solution was then poured into water when the nitro compound separated. The yield was generally about 85 per cent of the theoretical. The hydantoin was purified by crystallization from boiling acetic acid and separated on cooling as pale-yellow prisms, which melted at  $238^{\circ}$  to  $240^{\circ}$  with decomposition. The compound is soluble in hot alcohol, difficultly soluble in acetone and insoluble in ether.

ANALYSIS (Kjeldahl)	Calculated for $C_{16}H_{17}O_4N_3$	Found		
		I	II	III
N	17.87	17.87	17.81	17.58

*Reduction of 4-Paranitrobenzylhydantoin to 4-Para-aminobenzylhydantoin*

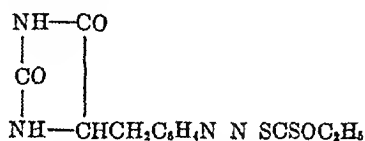
This amino hydantoin was obtained in the form of its hydrochloride by reduction of this nitro compound, in alcohol, with tin and hydrochloric acid. It was separated from tin in the usual

<sup>18</sup> Wheeler and Hoffman *loc cit*

manner and obtained in the form of prisms, which melted at  $255^{\circ}$  with decomposition. A mixture of this salt and the hydrochloride obtained by reduction of nitrobenzalhydantoin (above) melted at the same temperature.

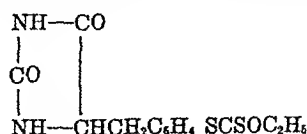
ANALYSIS	Calculated for $C_{10}H_{11}O_2N_2 \text{ HCl}$	Found
N	17.39	17.36
Cl	14.70	14.61

*4-Paradiazobenzylhydantoin Ethylxanthogenate*



The hydrochloride of 4-aminobenzylhydantoin was dissolved in dilute hydrochloric acid containing exactly one molecular proportion of hydrochloric acid and then diazotized at  $0^{\circ}$  to  $5^{\circ}$  by addition of the required amount of sodium nitrite. An excess of potassium xanthogenate was then dissolved in water and the solution added slowly to the diazonium solution. The diazo-xanthogenate separated at once as a voluminous yellow precipitate and was separated by suction filtration and washed with cold water. This compound is extremely unstable and gradually loses its nitrogen, being converted into the ethylxanthogenate of benzylhydantoin described below. No definite decomposition point could be assigned to this compound. After drying in a desiccator over sulphuric acid for one day it decomposed at about  $80^{\circ}$ , on the second day at  $115^{\circ}$  and after drying one week the decomposition point was  $140^{\circ}$  to  $150^{\circ}$ .

*Paraethylxanthogenate-4-benzylhydantoin*

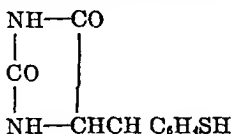


This compound was formed when the preceding diazonium compound was allowed to stand at ordinary temperature or when quickly heated at  $90^{\circ}$ . It was obtained as a brown powder, which

melted, without purification, at 150° with effervescence. It was purified for analysis by crystallization from methyl alcohol and separated in plates, which melted at about 170° with effervescence.

ANALYSIS (Kjeldahl)	Calculated for $C_{12}H_{14}O_2N_2S_2$	Found	
		I	II
N	9.03	9.06	9.01

*Thiotyrosinehydantoin*



This compound is formed by saponification of the above xanthogenate. This is accomplished not only by treatment with alkali but also by digestion with water. In fact it is not necessary to isolate the xanthogenate for the preparation of the hydantoin but to heat the diazonium compound in its own reaction fluid. Nitrogen is evolved, the xanthogenate is decomposed and thiotyrosinehydantoin is formed smoothly in one operation. The hydantoin is difficultly soluble in water or hydrochloric acid and separates, on cooling, in needles. It was purified for analysis by crystallization from 95 per cent alcohol and melted at 248° to 249°.

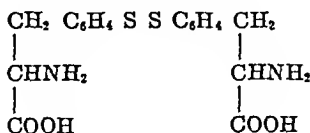
ANALYSIS (Kjeldahl)	Calculated for $C_{10}H_{10}O \text{ N S}$	Found		
		I	II	III
N	12.61	12.55	12.31	12.58

Molecular weight determination by the ebulliscopic method

- 0.2427 gram substance in 15.0 grams glacial acetic acid raised boiling point 0.23°
- 0.3636 gram substance in 15.0 grams glacial acetic acid raised boiling point 0.3°

	Calculated for $C_{10}H_{10}O_2N_2S$	Found	
		I	II
M. W.	222	178.	204

*Disulphide of Thiotyrosine*



Three grams of thiotyrosinohydantoin were digested with 50 grams of barium hydroxide in 100 cc of water for twenty hours. Barium carbonate was formed and ammonia was evolved. The barium was precipitated as sulphate by addition of a slight excess of sulphuric acid and the clear solution concentrated to a small volume. On cooling, the sulphate of this disulphide separated. It was purified by recrystallization from dilute sulphuric acid and separated in minute, colorless prisms arranged in burrs. In order to obtain the free amino-acid the sulphate was digested with water, when the salt underwent dissociation and the disulphide separated. This compound is extremely insoluble in water and melts at  $278^{\circ}$  with decomposition. It does not give Millon's test for tyrosine.

*Directions for Preparing this Disulphide directly from the Hydrochloride of 4-Para-aminobenzylhydantoin.* Forty grams of the aminobenzylhydantoin hydrochloride are carefully diazotized and the required amount of potassium xanthogenate added to the solution as described above. The yellow diazonium compound is then separated by filtration on a Buchner funnel, washed well with cold water and then transferred with water to a large Kjeldahl flask. The mixture is then warmed carefully to expel the nitrogen and finally boiled for thirty hours with 5 parts of barium hydroxide dissolved in hot water. In this manner the hydantoin ring is completely hydrolyzed, the xanthogenate is decomposed and the disulphide of thiotyrosine is formed. The barium carbonate is then filtered off, washed with water and the filtrate combined with sufficient dilute sulphuric acid to precipitate the excess of barium and combine with the disulphide of thiotyrosine to form the sulphate. This acid filtrate is then concentrated on the steam bath until the sulphate begins to separate from the hot solution and then cooled. Most of the sulphate deposits here and is separated by filtration. In order to obtain the pure amino-acid this salt is then dissociated by warming with water. More of the acid is obtained by cautiously adding ammonia to the sulphuric acid filtrate until the solution is distinctly alkaline and finally acidifying with acetic acid. The solution is then evaporated to dryness and the amino-acid separated from inorganic and organic salts by trituration with cold water. The yield of the amino-acid is about 90 per cent of the theoretical. It can be

purified by recrystallization from hot water. It is, however, extremely insoluble in this solvent.

Carbon and hydrogen determinations. 0.1611 gram substance gave 0.3244 gram  $\text{CO}_2$  and 0.0780 gram  $\text{H}_2\text{O}$ .

Sulphur determination (Carius). 0.1038 gram substance gave 0.1250 gram  $\text{BaSO}_4$ . Nitrogen determinations (Kjeldahl).

	Calculated for $\text{C}_{18}\text{H}_{17}\text{O}_4\text{N}_2\text{S}_2$	I	II	Found III	IV	V
C	55.10	54.92				
H	5.10	5.37				
N	7.14		7.09	7.14	7.15	
S	16.33					16.49

*The Properties of the Disulphide of Thiotyrosine.* This compound does not give the reaction of Adamkiewicz, Millon's test nor the biuret reaction. It likewise gives no blue color with ferric chloride solution. Its behavior towards concentrated sulphuric acid is, however, very characteristic. When the amino-acid or any of its salts is dissolved in this reagent and the solution is heated a beautiful purple color is produced. This color is destroyed by dilution of the acid solution with water. In fact this color test for thiotyrosine is as delicate and characteristic for thiotyrosine as the Millon's test is characteristic for tyrosine. Furthermore the characteristic test for each acid is not inhibited by the presence of the other acid. This observation is of importance because it is thus possible to test tyrosine for the presence of small traces of thiotyrosine. If found as a cleavage product of proteins it would probably accompany tyrosine because of its insolubility in water. The acid melts with decomposition at about  $278^\circ$  depending on the mode of heating. It is practically insoluble in all the common organic solvents except acetic acid. It is difficultly soluble in boiling water and separates, on cooling, in corpuscular crystals resembling in crystalline form that of isobarbituric acid. It combines with picric acid, hydrochlorplatinic acid, mercury chloride and phosphotungstic acid forming insoluble salts. No one of these, however, were characteristic and therefore were not examined. The acid dissolves in cold aqueous ammonia, sodium hydroxide and sodium carbonate solutions.

*Sulphate of the Disulphide of Thiotyrosine,*  $\text{C}_{18}\text{H}_{20}\text{O}_4\text{N}_2\text{S}_2 \cdot \text{H}_2\text{SO}_4$ . This salt crystallizes from dilute sulphuric acid in tufts of micro-

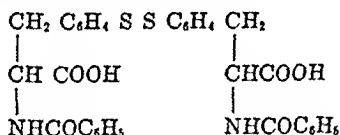
scopic crystals It undergoes dissociation when dissolved in water and the amino-acid separates It was dried for analysis in a vacuum desiccator over sulphuric acid and calcium chloride

ANALYSIS (Kjeldahl)	Calculated for	Found
	$C_{13}H_{17}O_4N_2S_2 \cdot H_2SO_4$	
N	5.72	5.85

*Hydrochloride*,  $C_{13}H_{20}O_4N_2S_2 \cdot 2HCl$  This salt crystallizes from hydrochloric acid in burrs of minute needles and decomposes at  $278^\circ$  This salt likewise undergoes dissociation when dissolved in water It was dried for analysis in a desiccator over potassium hydroxide

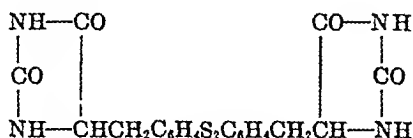
ANALYSIS	Calculated for	Found	
	$C_{13}H_{20}O_4N_2S_2 \cdot 2HCl$	i	ii
Cl	15.27	15.28	15.04

*Dibenzoyl Derivative of the Disulphide of Thiotyrosine,*



Sixty-three hundredths of a gram of anhydrous sodium carbonate was dissolved in 15 cc of water and 1 gram of the disulphide suspended in the solution Two molecular proportions of benzoylchloride were then added and the mixture shaken until the odor of benzoylchloride entirely disappeared The benzoyl compound was formed and separated as a colorless, crystalline solid After filtering, washing with dilute hydrochloric acid and finally with water, the compound was then dried at  $100^\circ$  The crude product was then digested with petroleum ether to remove benzoic acid and dried at  $100^\circ$  The yield was 1.5 grams The acid crystallizes from glacial acetic acid in prisms or clusters of needles and decomposes at about  $200^\circ$  with effervescence A mixture of this compound and the disulphide of thiotyrosine melted at  $179^\circ$  When digested with hydrochloric acid the benzoyl compound is decomposed and the disulphide of thiotyrosine, melting at  $278^\circ$ , is formed

ANALYSIS (Kjeldahl)	Calculated for $C_{27}H_{31}O_4N_2S_2$	Found	
		I	II
N	4.59	4.70	4.66

*Hydantoin of Thiotyrosine Disulphide,*

This compound was formed by the action of potassium cyanate on the hydrochloride of thiotyrosine disulphide. It could not be obtained in a crystalline condition and decomposed at about  $278^\circ$ .

ANALYSIS (Kjeldahl)	Calculated for $C_{20}H_{19}O_4N_4S_2$	Found
N	12.6	12.44

*Thiotyrosine*

This acid is formed by reduction of its disulphide with tin and hydrochloric acid in alcoholic solution. After the reduction was complete, the tin and excess of hydrochloric acid were removed in the customary way and the acid solution of the amino-acid concentrated and cooled. The hydrochloride of thiotyrosine separated. The yield was good and in one experiment we obtained 5.7 grams of the salt from 5 grams of the disulphide. This salt crystallized from 20 per cent hydrochloric acid in tufts of colorless, pointed prisms, which melted at  $249^\circ$  with effervescence. It was dried for analysis in a desiccator over potassium hydroxide.

ANALYSIS	Calculated for $C_9H_{11}O_2NS \cdot HCl$	Found	
		I	II
Cl	15.20	15.17	15.28

In order to obtain thiotyrosine this hydrochloric acid salt was dissolved in a small volume of water and ammonia cautiously added to combine with the hydrochloric acid. Thiotyrosine separated as an amorphous, colorless solid. It was separated as usual,

quickly washed with water and dried in a desiccator over sulphuric acid or calcium chloride. It melted without further purification at about  $250^{\circ}$  to a yellow oil with effervescence. This melting varies according to the mode of heating.

ANALYSIS Carbon and hydrogen determinations 0.1308 gram substance gave 0.2631 gram  $\text{CO}_2$  and 0.0674 gram  $\text{H}_2\text{O}$ .

Sulphur determination (Carius) 0.1134 gram substance gave 0.1314 gram  $\text{BaSO}_4$ .

Nitrogen determination (Kjeldahl)

	Calculated for $\text{C}_9\text{H}_{11}\text{O}_2\text{NS}$	I	Found II	III
C	54.82	54.59		
H	5.58	5.73		
S	16.24		15.87	
N	7.11			7.04

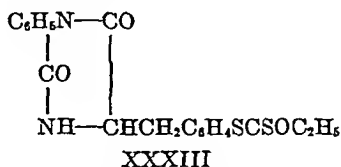
*Properties of Thiotyrosine* Thiotyrosine is practically insoluble in methyl or ethyl alcohol, petroleum ether, acetone, benzene and carbon bisulphide. It dissolves in hot water and glacial acetic acid. It cannot, however, be purified by recrystallization from water because of its instability. It slowly undergoes oxidation under these conditions and, on cooling the solution, the disulphide or a mixture of the disulphide and thiotyrosine deposits. The acid melts at  $249^{\circ}$  to  $250^{\circ}$  with decomposition while the disulphide melts at  $279^{\circ}$ . It does not give Millon's test for tyrosine, and reacts with sulphuric acid giving the same purple color as is formed by action of this acid on its disulphide. Attempts to prepare a characteristic benzoyl derivative were unsuccessful.

*The Preparation of Thiotyrosine from 1-Phenyl-4-para-aminobenzylhydantoin* Seven grams of 1-phenyl-4-para-aminobenzylhydantoin were dissolved in dilute hydrochloric acid and converted into its diazomium salt by addition of the required amount of sodium nitrite. An excess of potassium xanthogenate was then dissolved in water and this solution added slowly to the cold diazomium solution. The xanthogenate (XXXI) separated at





once as a bulky, yellow solid. This was then separated by filtration, washed with water and finally suspended in 200 cc of water in a Kjeldahl flask. On warming this mixture nitrogen was evolved and the corresponding xanthogenate (XXXIII) formed. This



compound was not isolated, but 75 grams of crystallized barium hydroxide were then added and the mixture boiled on a sand bath, with reflux condenser, for thirty hours. Barium carbonate was formed and aniline and volatile sulphur compounds were evolved. The aniline was expelled by distillation with steam, the barium carbonate filtered off and the excess of barium precipitated from the filtrate by addition of an excess of dilute sulphuric acid. On concentrating this acid solution and cooling the sulphate of the disulphide of thiotyrosine separated. The disulphide was obtained in the manner described above by dissociation of this salt with water. It crystallized from hot water in tufts of minute crystals which melted at 278°. When mixed with some disulphide, prepared from tyrosine hydantoin, this melting point was not altered. The acid also gave the characteristic purple color when warmed with concentrated sulphuric acid. The yield was 65 per cent of the theoretical. Two grams of this disulphide were reduced to thiotyrosine by means of tin and hydrochloric acid. After removal of the tin the acid was then obtained in the form of its hydrochloric acid salt, which melted at 248° with effervescence. It was dried for analysis in the air.

ANALYSIS	Calculated for	Found
	$\text{C}_7\text{H}_{11}\text{O}_2\text{NS HCl}$	
Cl	15.20	15.04

# A MODIFICATION OF RITTER'S METHOD FOR THE QUANTITATIVE ESTIMATION OF CHOLESTEROL

BY HARRY I CORPER

(From the Otho S A Sprague Memorial Institute and the Pathological Laboratory of the University of Chicago)

(Received for publication, June 7, 1912)

As was pointed out in previous papers<sup>1</sup> the quantitative determination of total cholesterol in small amounts by the Ritter method was found to be inadequate, not only by myself but also by other authors<sup>2</sup>. The source of inaccuracy of the method was found to lie in the fact that an excess of sodium alcoholate prevents a quantitative extraction of cholesterol. If this source of error were eliminated it was hoped that we might have a method at our disposal which could be used for the quantitative estimation of cholesterol, as, from a chemical standpoint, Ritter's method seemed best adapted for avoiding the troublesome interference occasioned by the formation of soaps in shaking out the cholesterol by means of ether. Another point of importance in the quantitative determination of cholesterol was the fact that in the saponification we must be assured that sufficient sodium alcoholate has been used to completely saponify all the fats and the cholesterol-esters. In the original Ritter method we are restricted in the use of the saponifying agent, as, when this is used even in slight excess, a considerable loss of cholesterol may result. The aim of this paper, then, is to point out a way to surmount these difficulties and determine cholesterol gravimetrically within milligrams.

The gravimetric methods recommended for the determination of cholesterol are numerous but have been worked out mainly for determining large amounts, and in many cases have not been suffi-

<sup>1</sup> Corper *this Journal*, xi, p 37, 1912, xi, p 27, 1912

<sup>2</sup> Corper *Journ of Exp Med*, xv, p 429, 1912, Baldwin *this Journal*, iv, p 213, 1908, Waldemar Koch (personal interview) was unsuccessful in the use of Ritter's method on brain tissues

ciently well controlled to rule out all possibilities of error. Only such literature will be cited as has a direct bearing upon the work given in this paper.

Gérard and Verhaeghe<sup>3</sup> saponified the fats by means of alcoholic potash, passed carbon dioxide into this mixture, evaporated the saponified mixture to dryness in the presence of sand and extracted this mixture with anhydrous ether. The ethereal extract was then fused with benzoic anhydride and cholesterol determined as cholesterol benzoate. All the figures are given on the basis of 100 grams, moist weight, so that it is impossible to say how accurate this method is. Gérard<sup>4</sup> examined 70.9 liters of urine for cholesterol by evaporating it to dryness after mixing with sand, extracting the dried mixture in a Soxhlet by means of anhydrous ether, the ethereal extract dissolved in alcohol saponified by means of potash, carbon dioxide then passed into this, the potassium carbonate filtered off, the filtrate evaporated to dryness and extracted by means of ether. This extract weighed 0.544 gram and contained 0.011 gram of impure cholesterol.

Where only small amounts of material are to be examined for cholesterol, not sufficient for a gravimetric analysis, the colorimetric methods may be of service. Two of these have been recommended, one on the basis of the Salkowski reaction and the other using the Liebermann reaction.

Weston,<sup>5</sup> in a thorough piece of work with pure cholesterol, found that the Salkowski reaction was serviceable for the quantitative determination of small amounts of pure cholesterol, the best colors for comparison being obtained between 0.1 mgm. and 0.25 mgm.

Grigaut<sup>6</sup> used the Liebermann reaction for the quantitative determination of cholesterol after extracting and saponifying. He says the reaction is best between 0.1 and 3.0 mgm. The method<sup>7</sup> is not serviceable when ischolesterol, etc., are present. In a second<sup>8</sup> modified method the author puts 2 cc. of serum, or 0.1 to 1.0 gram of fresh ground tissue, and 20 cc. of 1 per cent soda in 50 per cent alcohol into a 90 cc. flask. This is placed in a water bath for fifteen to twenty minutes, cooled and extracted with ether, the ether extract is evaporated and dissolved in chloroform for the test. By this means Grigaut says he obtains figures comparable to gravimetric figures.

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<sup>3</sup> Gérard and Verhaeghe *Journ de pharm et de chim*, III, serie 7, p 385, 1911

<sup>4</sup> Gérard *Compt rend soc biol*, LXX, serie 22, p 998, 1911

<sup>5</sup> Weston *Journ of Med Res*, XXVI, p 47, 1912

<sup>6</sup> Grigaut *Compt rend soc biol*, LXXIII, serie 16, p 791, 1910

<sup>7</sup> *Ibid*, LXXIII, serie 17, p 827, 1910

<sup>8</sup> *Ibid*, LXXI, serie 33, p 513 1911

## EXPERIMENTAL PART

Ritter<sup>9</sup> adds 100 cc of alcohol to 50 grams of fat, brings the mixture to a boil on a water bath and then adds to this 160 cc of sodium alcoholate described by Kossel and Kruger<sup>10</sup> The alcohol is then evaporated off on the water bath, salt is added in amount equivalent to about one and a half times the weight of fat taken (to prevent the extraction of soaps by ether) and enough water so that the residue goes into solution This is then dried on the water bath with constant stirring, and then at 80°C in a drying oven It is pulverized, put into a sulphuric acid desiccator for a short time, then into an extraction thimble and is extracted in a Soxhlet apparatus with ordinary ether for nine hours The ether extract is shaken out with water to remove glycerin, then dried, dissolved in hot alcohol, precipitated by means of water, the precipitate dried at 100° to 120°C, and weighed

Since, as shown in my previous paper, an excess of sodium alcoholate prevents thorough extraction of cholesterol from the salt mixture, and an excess is absolutely essential to guarantee complete saponification, it was found necessary to remove sodium alcoholate from the field of action and at the same time not to introduce a factor or a chemical which would liberate the fatty acids from soaps Fatty acids being ether-soluble our results would otherwise never be quantitative This difficulty was overcome by using carbon dioxide in order to form sodium hydrogen carbonate, which is weakly alkaline and cannot liberate the fatty acids from the soaps, whether it would likewise prevent a quantitative extraction of cholesterol from the salt mixture had to be determined For this purpose pure cholesterol was used and the entire process carried through as follows

Duplicate samples of cholesterol, 0.100 gram, were dissolved in 10 cc of absolute alcohol by heating on the water bath After complete solution 40 cc of 5 per cent sodium alcoholate were added and the mixture further heated for about fifteen to twenty minutes, cooled, allowed to stand over night (this was all done in a large beaker, 500 cc or 1 liter, Jena glass), then about 100 cc of water added and through this was passed a slow stream of carbon dioxide gas from a Kipp generator for from three to five hours The

<sup>9</sup> Ritter *Zeitschr f physiol Chem*, xxiv, p 461, 1903

<sup>10</sup> Kossel and Kruger *ibid*, xv, p 321, 1891

mixture was then warmed and poured into an evaporating dish and 20 to 30 grams of table salt added, the entire mixture evaporated to dryness on the water bath with stirring and, when dry, quantitatively transferred to a Soxhlet thimble, to be further dried at  $100^{\circ}\text{C}$  in a drying oven for an hour or two and then cooled in a desiccator. This mixture was extracted for seven to nine hours in a Soxhlet apparatus with ether (anhydrous), the ether extract (diluted to 100 to 300 cc) shaken out with three changes of distilled water (200 to 500 cc), the combined water again shaken out with about 50 to 100 cc of ether and this ether further washed with two more changes of water. The combined ethers were evaporated to dryness in a large beaker and then, when of small volume, transferred to a small weighed beaker, dried at  $100^{\circ}\text{C}$  and weighed. Recovered Experiment 1A, 0.104 gram, Experiment 1B, 0.101 gram.

Two other samples were analyzed, using 0.100 gram of pure cholesterol through which carbon dioxide had been passed for only about two and a half hours. Recovered Experiment 1C, 0.095 gram, Experiment 1D, 0.093 gram.

As a result of the above experiments we can say that carbon dioxide passed into the saponified mixture will successfully remove the sodium alcoholate so that it will not interfere with the quantitative extraction of cholesterol from the soap-sodium chloride mixture in the Soxhlet apparatus. In order to assure the removal of all the sodium alcoholate, however, the carbon dioxide should be passed into the mixture for at least three hours, preferably for five hours.

To determine whether cholesterol could be recovered quantitatively from large amounts of fat, the following experiments were carried out.

EXPERIMENT 2A. 0.100 gram of pure cholesterol was mixed with 8 cc of olive oil, the mixture dissolved in 20 cc of absolute alcohol and saponified with 50 cc of 5 per cent sodium alcoholate, allowed to stand over night, carbon dioxide added to saturation, etc., as above. Recovered 0.147 gram of cholesterol.

EXPERIMENT 2B. Duplicate of Experiment 2A, with the exception that 5 cc of olive oil were used. Recovered 0.129 gram of cholesterol.

Unfortunately the olive oil used contained cholesterol but if we consider the amount used the results are quantitative, as in the first case, from 8 cc of olive oil, 47 mgm of cholesterol were recovered, and from 5 cc, 29 mgm, which is about five-eighths of 47 mgm.

From these experiments we can conclude that by this method cholesterol may be recovered quantitatively even from fatty mixtures poor in cholesterol.

Determinations on different tissues were made, using human liver and dog's spleen

*Analysis of human liver for cholesterol*<sup>11</sup>

EXPERIMENT 3 3 77 grams of the fatty extract of human liver (tissue extracted in a Wiley apparatus by means of hot alcohol and hot ether), divided into two equal parts after solution in hot absolute alcohol, was analyzed for cholesterol by the above method, using 10 cc of sodium alcoholate for saponification Recovered A, 0 103 gram, B, 0 100 gram of cholesterol

EXPERIMENT 4 2 38 grams of fatty extract from human liver, divided into two equal parts, was analyzed, using for saponification 40 cc of sodium alcoholate Recovered A, 0 088 gram, B, 0 089 gram of cholesterol

EXPERIMENT 5 In order to see whether there was any marked difference in duplicates when using on one (A) 10 cc of sodium alcoholate and on the other (B) 40 cc of sodium alcoholate, 1 57 grams of the alcohol-ether extracts of human liver were divided into equal parts and analyzed Recovered A, 0 066 gram, B, 0 069 gram of cholesterol

The thorough agreement in these results can further be seen when they are tabulated to show the percentage of cholesterol in the three different livers, Experiments 3, 4 and 5, on the basis of these analyses The figures express percentages of dry weight of liver tissue

<i>Experiment 3</i>		<i>Experiment 4</i>		<i>Experiment 5</i>	
A	1 95	A	1 93	A	1 90
B	1 90	B	1 93	B	1 96

As a result of these analyses we can conclude that the above method is applicable to the quantitative determination of cholesterol in the fats of the liver and that an excess of sodium alcoholate may be used to insure complete saponification, as the passage of carbon dioxide prevents the detrimental action of sodium alcoholate later in the process and does not affect the yield

*Analysis of dog spleen for cholesterol*

EXPERIMENT 6 70 grams of dog spleen, moist weight, were thoroughly ground up and extracted with hot alcohol and ether in a Greene extraction apparatus,<sup>12</sup> and the total fats, 2 98 grams, dissolved in 250 cc of hot abso-

<sup>11</sup> The fats for these analyses (Experiments 3, 4 and 5) were obtained from Mr E, R Long, to whom I am indebted for their use in connection with this work The description of these livers and complete chemical analyses will be published by him at a later period

<sup>12</sup> Greene this *Journal*, vii, p 503, 1910

lute alcohol Two 100 cc portions of this (A and B) were taken for gravimetric analyses for cholesterol and 50 cc for the determination of cholesterol by Weston's method<sup>13</sup>

A This sample of fat was saponified by means of 10 cc of 5 per cent sodium alcoholate and the resultant ether extract from the Soxhlet apparatus was cloudy and yellow On shaking out the ether with water there was found to be present a large amount of soap which frothed and also prevented a rapid separation of the ether water layers The yield was also higher than in the following experiment, a fact which may be accounted for by the difficulty in thorough separation Recovered 0.177 gram of cholesterol

B Duplicate of A but saponified by means of 40 cc of 5 per cent sodium alcoholate The ether-water separation was rapid and perfect, absolutely no frothing, and even the shaking water was clear, as was also the supernatant ether layer Recovered 0.162 gram of cholesterol

The product recovered from the above tissues (liver and spleen) was of a pale yellow color and was composed of typical crystals which gave the ordinary cholesterol reactions

As a result of the experiments on the spleen we can say that an excess of sodium alcoholate, when using this method, is rather an advantage since it prevents the possibility of incomplete saponification and soaps are less liable to be extracted by the ether Numerous other gravimetric analyses for cholesterol have been carried out by this method, but mainly on cholesterol-rich materials, and the method has proved satisfactory in these cases also On the basis of this work the following method is recommended for the quantitative gravimetric determination of total cholesterol (cholesterol-like compounds) in tissues

*The sample of tissue, about 30 grams or more, is thoroughly extracted by means of hot absolute alcohol, then with ether, the extract is then evaporated to dryness, taken up in about 200 cc of hot absolute alcohol, divided into two equal parts for duplicates, evaporated to 10 to 20 cc, saponified by means of an excess of 5 per cent sodium alcoholate (20 to 30 cc should be enough for the fats from 15 grams of moist tissue) and kept on the water bath for about fifteen to twenty minutes or until saponification is complete, and then kept at room temperature over night This may all be carried out in a large Jena glass beaker of about 500 cc capacity About 100 cc of distilled water is added and a moderate stream of carbon dioxide passed into this mixture long enough*

<sup>13</sup> Weston *loc cit*

to insure complete saturation (three to five hours) The mixture is warmed and poured into a large evaporating dish and 20 to 30 grams of ordinary table salt added and the entire mixture evaporated to dryness on the water bath, with occasional stirring (stirring is only necessary toward the end of the drying process to prevent too much lumping which interferes with thorough drying and satisfactory pulverizing) The dried residue is now pulverized in a mortar and put into an extraction thimble and dried further for an hour or two at  $100^{\circ}\text{C}$  in a drying oven, at the end of which time it is allowed to cool in a desiccator When cool it is extracted in a Soxhlet apparatus by means of anhydrous ether (distilled over sodium) for from seven to nine hours At the end of this time the ether extract is transferred to a large separatory funnel, 500 cc or 1 liter capacity, diluted to about 100 cc with ordinary ether (C P) and shaken out with three changes of distilled water (200 to 500 cc), this total watery extract to be again shaken out with another portion of ordinary ether (C P), about 100 cc, and the ether washed with two changes of distilled water The total ether extracts, which contain the cholesterol and cholesterol-like substances of the tissue, are now evaporated to dryness, first by placing *under* an electric bulb (16 C P) and finally dried for an hour or two at  $100^{\circ}\text{C}$ , cooled in a desiccator and weighed

### *Colorimetric comparisons*

Weston's description of his colorimetric method applies only to pure cholesterol, it was therefore thought desirable to make a few tests with the available material from tissue extracts to see whether the colorimetric method would agree with the gravimetric method For this purpose the remaining 50 cc of the above spleen fats (Experiment 6) were divided into two 25 cc portions, one portion evaporated to dryness and dissolved in chloroform and the other saponified by means of 2 cc of 5 per cent sodium alcoholate, treated with carbon dioxide, evaporated to dryness, extracted with chloroform, filtered and diluted to a known volume These chloroform solutions were then compared with standards made with pure cholesterol as directed by Weston The 0.1 mgm standard solutions agreed in color with the 0.2 cc of a 1:100 dilution of the fats from the spleen This represents a total of 0.5 gram in the entire 250



cc of absolute alcohol solution, as compared to about 0.4 gram obtained by the gravimetric method. The saponified and unsaponified fats agreed exactly in color.

Colorimetric analyses were also made upon the fats of the human livers used in Experiments 3, 4 and 5, with the following results.

The 0.15 mgm cholesterol standard agreed in color with 0.1 cc of a 1:100 dilution in chloroform of the fats of Experiment 3A, making a total of 150 mgm of cholesterol colorimetrically as compared to 103 mgm found gravimetrically.

The 0.15 mgm cholesterol standard agreed in color with 0.05 cc of a 1:100 dilution in chloroform of the fats of Experiment 4A, making a total of 300 mgm of cholesterol colorimetrically as compared to 88 mgm found gravimetrically. The 0.1 mgm cholesterol standard agreed in color with 0.05 cc of a 1:75 dilution in chloroform of the fats of Experiment 5A, making a total of 150 mgm of cholesterol colorimetrically as compared to 66 mgm found gravimetrically.

These colorimetric analyses of the fats of the spleen and liver do not correspond at all with the figures obtained by the gravimetric method.

#### SUMMARY

1. The error occasioned in the determination of cholesterol by the Ritter method by the use of an excess of sodium alcoholate in saponifying the fats, as reported in a previous paper,<sup>14</sup> has been successfully overcome by neutralizing the sodium alcoholate by means of carbon dioxide gas.

2. As a result of this a method is recommended for determining total cholesterol and cholesterol-like substances in tissues, which is accurate within milligrams, as determined by analysis of pure cholesterol, cholesterol in olive oil, cholesterol from the fats of the liver and from the fats of the spleen.

<sup>14</sup> Corper, *this Journal*, xi, p. 37, 1912.

# HYDANTOINS A NEW METHOD FOR THE SYNTHESIS OF PHENYLALANINE

THIRTEENTH PAPER

By TREAT B JOHNSON AND WILLIAM B O'BRIEN

(From the Sheffield Laboratory of Yale University)

(Received for publication, June 14, 1912)

Phenylalanine,  $C_6H_5CH_2CH(NH_2)COOH$ , has been synthesized by several different methods. Arranged chronologically, they are as follows:

*Method 1* By the action of ammonia on the nitrile of  $\alpha$ -hydroxy- $\beta$ -phenylpropionic acid and then hydrolysis of the resulting amino-nitrile<sup>1</sup>

*Method 2* By reduction of the oxime of phenylpyruvic acid<sup>2</sup>

*Method 3* Benzaldehyde is condensed with hippuric acid when the lactimide of benzoylamino-cinnamic acid is formed. This is then hydrolyzed and reduced to the benzoyl derivative of phenylalanine and the latter converted by hydrolysis into the amino-acid<sup>3</sup>

*Method 4* By heating cinnamic acid or its esters with hydroxylamine<sup>4</sup>

*Method 5* The benzyl group is introduced into diethyl phthalimidomalonate, by alkylation, and the resulting phthalimido derivative then converted into the amino-acid by hydrolysis<sup>5</sup>

<sup>1</sup>Erlenmeyer and Lipp *Ann d Chem (Liebig)*, ccix, p 194, *Ber d deutsch chem Gesellsch*, xv, p 1006

<sup>2</sup>Erlenmeyer *Ann d Chem (Liebig)*, cclxxi, p 169, Knoop and Hoessli *Ber d deutsch chem Gesellsch*, xxxix, p 1477

<sup>3</sup>Plöchl *Ber d deutsch chem Gesellsch*, lvi, p 2815, lvii, p 1623, Erlenmeyer *Ann d Chem (Liebig)*, cclxxv, pp 3, 18

<sup>4</sup>Posner *Ber d deutsch chem Gesellsch*, xxxvi, p 4312

<sup>5</sup>Sørensen *Zeitschr f physiol Chem*, xlv, p 448

*Method 6* From brombenzylmalonic acid This is converted into the monobasic acid by heating and the latter then heated with ammonia<sup>6</sup>

*Method 7* From benzalhydantoin, which is obtained by condensation of benzaldehyde with hydantoin This hydantoin is then converted into the hydantoin of phenylalanine, by reduction, and the amino-acid then obtained by hydrolysis with barium hydroxide<sup>7</sup>

The various changes involved in these seven different methods of synthesis are represented by the formulas given on the following page

Of these different methods of preparation, method 7 is undoubtedly the most practical when hydantoin is available It requires only three operations (two if hydriodic acid is used as a reducing agent), and the yield of amino-acid is excellent The only drawback is the cost of the hydantoin While the preparation of this reagent is not difficult, nevertheless its synthesis involves the preparation of glycocoll or its ethyl ester and also the use of potassium cyanate, which is an unstable salt and oftentimes is not available in large quantity because of its cost In this paper we shall describe a method for the synthesis of phenylalanine, which does not require the use of hydantoin and hydriodic acid We use potassium thiocyanate, instead of potassium cyanate<sup>8</sup>, and tin and hydrochloric acid as the reducing agent

In a previous paper from this laboratory,<sup>9</sup> it has been shown that 2-thio-3-benzoylhydantoin (II) is formed smoothly by the action of potassium thiocyanate on hippuric acid (I) We now find that this hydantoin condenses with benzaldehyde forming practically a quantitative yield of the corresponding benzalhydantoin (III) When this acyl hydantoin (III) is warmed with hydrochloric acid the benzoyl group is removed and the same benzalthiohydantoin (VI) is formed as is obtained by condensing benzaldehyde with 2-thiohydantoin<sup>10</sup> (VII) The isomeric 1-

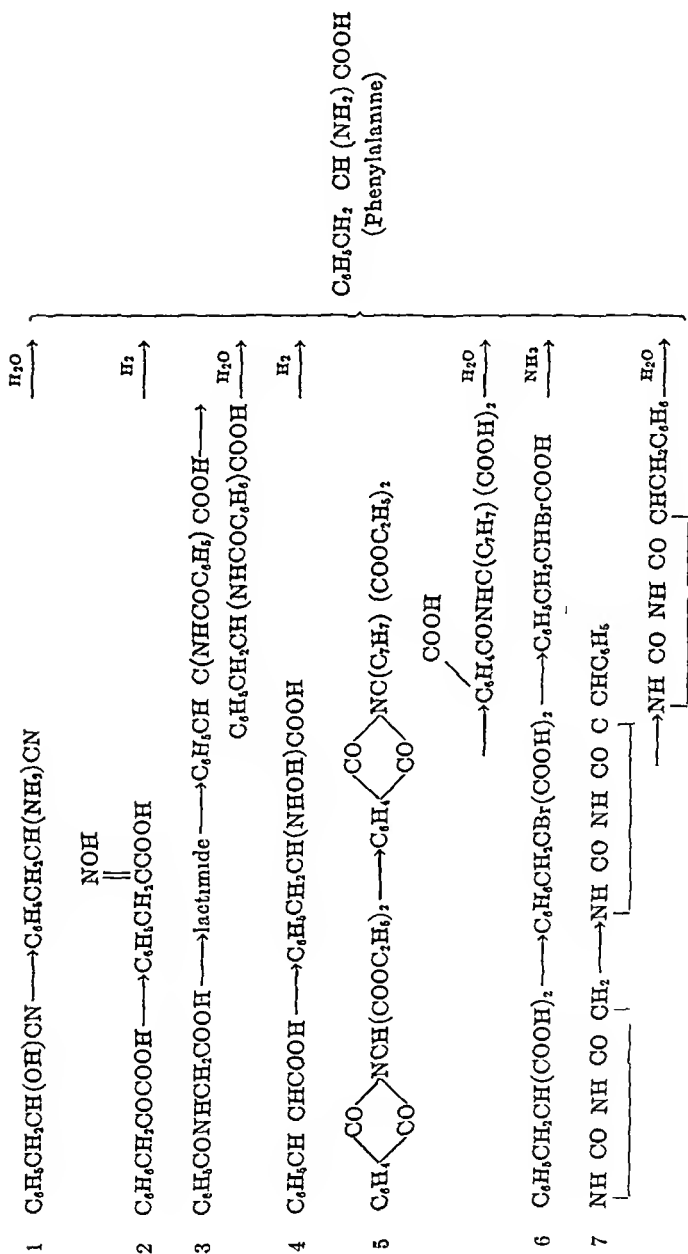
<sup>6</sup> Fischer *Ber d deutsch chem Gesellsch*, xlvii, p 3064

<sup>7</sup> Wheeler and Hoffman *Amer Chem Journ* xlv, p 368

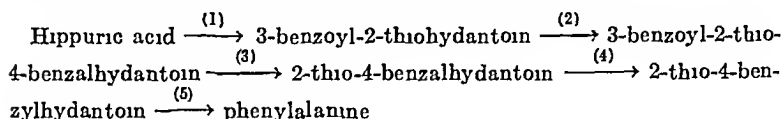
<sup>8</sup> Potassium cyanate is about nine times as expensive as potassium thiocyanate (Kahlbaum's quotations)

<sup>9</sup> Johnson and Nicolet *Journ Amer Chem Soc*, xxxiii, p 1973

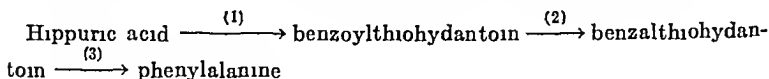
<sup>10</sup> Wheeler, Nicolet and Johnson *Amer Chem Journ*, xlv, p 456, Johnson and Nicolet, *loc cit*



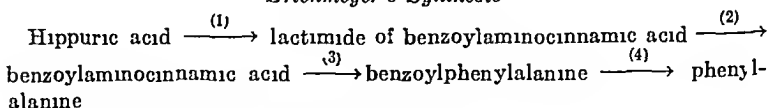
benzoyl-2-thio-4-benzalhydantoin has also been prepared<sup>11</sup> The thiohydantoin (VI) is desulphurized by digestion with chloracetic acid, in aqueous solution, giving the corresponding hydantoin (V) and is also transformed quantitatively, by reduction with sodium amalgam, into the thiohydantoin of phenylalanine (IX) This thiohydantoin (IX) is likewise easily desulphurized by means of chloracetic acid, and, by hydrolysis with boiling barium hydroxide solution, is transformed quantitatively into phenylalanine (X) Using sodium amalgam as the reducing agent, this synthesis therefore requires five operations as follows



We have now made the interesting observation that it is not necessary to isolate 3-benzoyl-2-thio-4-benzalhydantoin (III) or 2-thio-4-benzylhydantoin (IX) The condensation product (III) is at once converted into benzalthiohydantoin (VI) and the latter is then reduced with tin and hydrochloric acid In this manner the double bond is not only reduced smoothly, but the hydantoin ring is also destroyed and phenylalanine is formed quantitatively in one operation Therefore by using tin and hydrochloric acid as the reducing agent our synthesis requires only *three* operations and potassium thiocyanate can be used in place of potassium cyanate The starting point in our new synthesis and in Erlenmeyer's (method 3) is hippuric acid, but the latter condenses the acid directly with the aldehyde, while we first transform the acid into the thiohydantoin (II) and then apply an aldehyde condensation Erlenmeyer's synthesis involves four operations while ours requires only three as follows

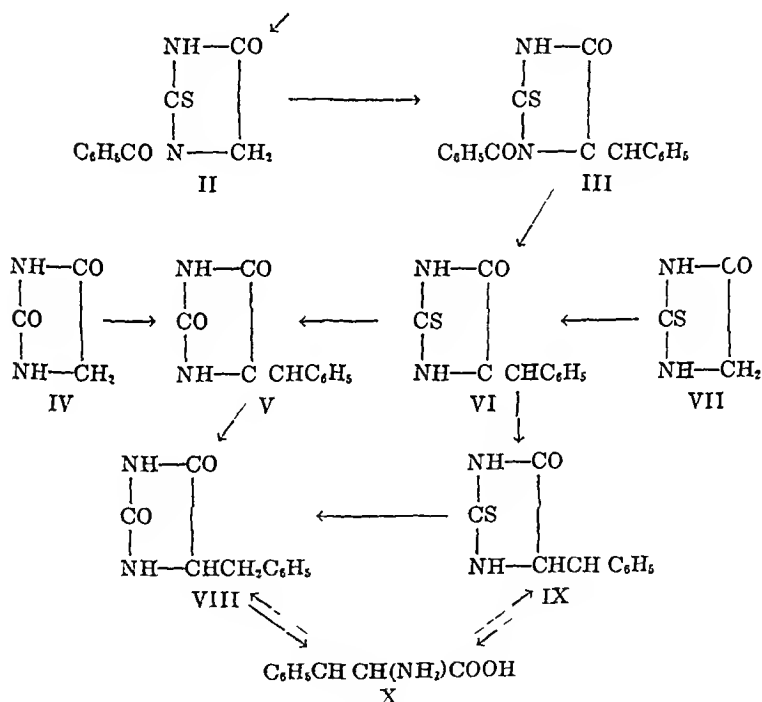
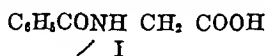


#### *Erlenmeyer's Synthesis*



<sup>11</sup> Wheeler, Johnson and Nicolet *loc cit*

If hippuric acid is not available glycocholl can be converted smoothly into 2-thiohydantoin by the action of potassium thiocyanate<sup>12</sup> and the benzalthiohydantoin prepared by condensation with benzaldehyde. These various transformations are represented by the following structural formulas



We shall continue our investigations on thiohydantoin

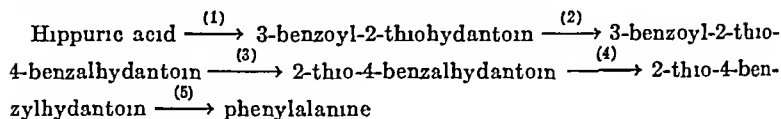
#### EXPERIMENTAL PART

The 3-benzoyl-2-thiohydantoin used in this work was made, according to the directions of Johnson and Nicolet, by the action of potassium thiocyanate on hippuric acid<sup>13</sup>

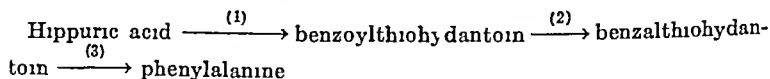
<sup>12</sup> Johnson and Nicolet *loc cit*

<sup>13</sup> *Loc cit*

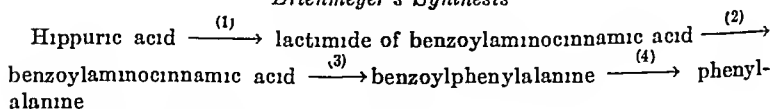
benzoyl-2-thio-4-benzalhydantoin has also been prepared<sup>11</sup> The thiohydantoin (VI) is desulphurized by digestion with chloracetic acid, in aqueous solution, giving the corresponding hydantoin (V) and is also transformed quantitatively, by reduction with sodium amalgam, into the thiohydantoin of phenylalanine (IX) This thiohydantoin (IX) is likewise easily desulphurized by means of chloracetic acid, and, by hydrolysis with boiling barium hydroxide solution, is transformed quantitatively into phenylalanine (X) Using sodium amalgam as the reducing agent, this synthesis therefore requires five operations as follows



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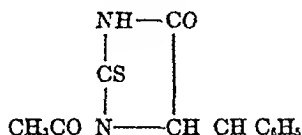
#### *Erlenmeyer's Synthesis*



<sup>11</sup> Wheeler, Johnson and Nicolet *loc cit*

*Desulphurization of 2-Thio-4-benzalhydantoin*

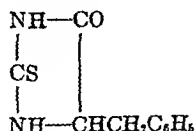
This hydantoin was converted quantitatively into 4-benzalhydantoin<sup>16</sup> by digestion with a 20 per cent aqueous solution of chloroacetic acid. The hydantoin crystallized from alcohol in needles which melted at 220°

*2-Thio-S-acetyl-4-benzylhydantoin*

Two grams of phenylalanine and 1.5 grams of anhydrous potassium thiocyanate were dissolved in a mixture of 8 cc of acetic anhydride and 1 cc of glacial acetic acid and the solution warmed on the steam bath for thirty minutes. A yellow solution was obtained, which was diluted with about five volumes of water. This hydantoin separated as an oil, which soon crystallized in the form of plates. It was purified by crystallization from alcohol and melted at 257°. The yield was quantitative.

## ANALYSIS (Kjeldahl)

	Calculated for $\text{C}_{12}\text{H}_{13}\text{O}_2\text{N}_2\text{S}$	Found
N	11.47	11.35

*2-Thio-4-benzylhydantoin*

This hydantoin was obtained by hydrolysis of the preceding acetyl thiohydantoin with hydrochloric acid. It was purified by crystallization from dilute alcohol and separated in needles, which melted at 185°.

<sup>16</sup> Wheeler and Hoffman *Amer Chem Journ*, xiv, p 371



## ANALYSIS (Kjeldahl)

N	Calculated for $C_{10}H_{10}ON_2S$	Found	
		I	II
	13.59	13.52	13.40

This same hydantoin is also formed smoothly by reduction of 2-thio-4-benzalhydantoin with sodium amalgam

*Desulphurization of 2-Thio-4-benzylhydantoin*

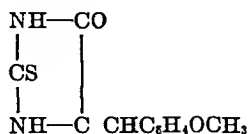
This hydantoin was converted quantitatively into the hydantoin of phenylalanine by digesting this for one hour with a 25 per cent solution of chloracetic acid. It crystallized from water in prisms, which melted at  $190^\circ$  to a clear oil.

*The Formation of Phenylalanine by Reduction of 2-Thio-4-benzalhydantoin with Tin and Hydrochloric acid*

Eight and five-tenths grams of 2-thio-4-benzalhydantoin and 10 grams of tin were suspended in dilute alcohol and hydrochloric acid gas passed into the alcohol (warm) until all the tin and hydantoin dissolved. After allowing to stand for from eight to ten hours the clear solution was then evaporated to dryness and the residue dissolved in water. The tin was removed completely by precipitation as sulphide and the filtrate again concentrated to a small volume and the hydrochloric acid neutralized by addition of ammonia. Pure, colorless phenylalanine separated and melted at  $260-262^\circ$ . The yield was nearly quantitative.

## ANALYSIS (Kjeldahl)

N	Calculated for $C_9H_{11}O_2N$	Found
	8.50	8.80

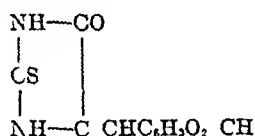
*2-Thio-4-anisalhydantoin*

Six and five-tenths grams of 3-benzoyl-2-thiohydantoin, 11 grams of anhydrous sodium acetate and 4 grams of anisic aldehyde

were dissolved in 22 cc of glacial acetic acid and the solution heated in an oil bath at 150° for two hours. The condensation product separated on cooling. After trituration with an excess of water the crude hydantoin was separated by filtration. This was dissolved in alkali and the warm solution acidified with hydrochloric acid when the above hydantoin separated. It was purified by crystallization from 50 per cent alcohol and melted at 257° to a black oil with decomposition.

## ANALYSIS (Kjeldahl)

	Calculated for $C_{11}H_{10}O_2NS$	Found
N	11.91	12.0

*2-Thio-4-piperonalhydantoin*

From piperonal and 2-thio-3-benzoylhydantoin. It is very insoluble in alcohol, water and glacial acetic acid and decomposes when heated above 285° giving a dark oil. The hydantoin dissolves in concentrated sulphuric acid giving a deep purple solution.

## ANALYSIS (Kjeldahl),

	Calculated for $C_{11}H_9O_2N_2S$	Found
N	11.2	11.06



## STUDIES IN BACTERIAL METABOLISM III

B<sub>1</sub> ARTHUR I KENDALL AND CHESTER J FARMER

(From the Laboratories of Biological Chemistry and Preventive Medicine  
and Hygiene, Harvard Medical School)

(Received for publication, June 15, 1912)

This work is a direct continuation of that described in previous communications<sup>1</sup>. In this series of experiments the sparing action of carbohydrate for protein is again quantitatively brought out. *B. proteus* and *B. coli* are the only organisms of those described here which have been previously investigated by us. The analytical results with these two strains bear out those obtained with the strains reported in the first and second papers of these studies.

*B. mesentericus*, a and b, are respectively two strains of the same organism. One of them was obtained from milk, the other was isolated from the feces of a young baby which had been fed upon this milk. Culturally the two strains are identical, chemically their proteolytic and fermentative powers are also practically identical as may be seen from an inspection of the table. Both of these organisms broke down about 25 per cent of the total protein nitrogen of the medium in which they were grown to ammonia in the sugar-free broth in seven days, while in the corresponding sugar broth, only 1.5 per cent of the total protein was broken down to ammonia in seven days. That is to say, more than sixteen times as much protein was broken down to ammonia in the sugar-free broth as was the case in the dextrose broth in the same length of time.

The growth curve of the diphtheria bacillus, shown by the rate and amount of ammonia formation in the sugar-containing and sugar-free broth, is quantitatively like that of the typhoid

<sup>1</sup> This *Journal* xii, pp 13, 19, 1912

and dysentery bacilli, and needs no further comment. It will be remembered that in sugar-free broth the diphtheria bacillus produces a strong extracellular toxin, while in broth containing utilizable sugar there is little or no toxin formed, the slight excess of ammonia formed in the sugar-free broth becomes significant when interpreted in the light of this remarkable difference in toxicity between purely protein broth and broth containing both protein and sugar which can be fermented by the diphtheria bacillus.

*Micrococcus aureus* (*Staphylococcus pyogenes aureus*) brings out a new factor in bacterial metabolism. It will be seen that this organism brings out a progressive acidity even in sugar-free broth. The bacteria described previously in this series of studies progressively form alkali in this medium, with the single exception of the Shiga bacillus. We believe that this acidity is due to the fermentation of the carbohydrate radical of the protein molecule (Witte's peptone) by this organism, *Micrococcus aureus*. Pick has shown that that fraction of Witte's peptone which may be salted out between 66 and 100 per cent saturation with ammonium sulphate contains a relatively large amount of a substance reacting chemically like a true carbohydrate. It would appear likely that the *Micrococcus aureus* acts selectively upon this carbohydrate fraction of the peptone molecule. This acid reaction produced in the so-called "sugar-free" broth increases progressively to the sixth day of growth. During this time the ammonia production increases very slowly, hardly more is produced than in the broth containing sugar, in fact. On the eighth day the reaction in the sugar-free broth becomes strongly alkaline, and this alkaline reaction increases in intensity to the end of the experiment. This abrupt change in reaction from acidity to alkalinity, together with the sudden increase in ammonia formation, can be explained satisfactorily on the assumption that the carbohydrate radical of the protein is practically exhausted on the sixth day, and that this exhaustion of the carbohydrate leaves the organism free to attack the protein both for structural and fuel purposes.

This result, therefore, perplexing as it seemed at first, appears in reality to furnish an unexpected confirmation of the theory that carbohydrate which is utilizable protects protein from bacterial attack to a considerable degree.

TABLE I  
Broth D

	DAYS	PLAIN BROTH			DEXTROSE BROTH		
		Free NH <sub>3</sub> as milligrams N per 100 cc	Ammonia N Total N	Reaction cc N acid per 100 cc	Free NH <sub>3</sub> as milligrams N per 100 cc	Ammonia N Total N	Reaction cc N acid per 100 cc
Control		11 90	per cent 5 30	+ 0 50	11 90	per cent 5 30	+ 0 75
B diphtheriae	1	11 90	5 30	0 00	11 90	5 30	+ 0 75
	3	12 95	5 80	0 00	12 60	5 61	+ 1 25
	6	13 30	5 94	0 00	12 95	5 80	+ 1 75
	8	13 65	6 10	- 0 25	11 90	5 30	+ 1 75
	10	16 80	7 50	- 0 75	13 30	5 94	+ 1 75
Micrococcus aureus	1	12 60	5 61	0 00	12 25	5 46	+ 1 75
	3	15 40	6 90	+ 0 50	14 00	6 25	+ 2 75
	6	17 85	7 96	+ 0 75	14 00	6 25	+ 2 75
	8	27 30	12 20	- 1 00	12 95	5 80	+ 2 75
	10	30 80	17 00	- 1 75	14 35	6 40	+ 2 50
Streptococcus pyogenes	1	12 25	5 46	0 00	11 55	5 16	+ 3 00
	3	12 25	5 46	+ 0 25	12 25	5 46	+ 4 50
	6	13 30	5 94	+ 0 25	12 25	5 46	+ 5 25
	8	12 95	5 80	+ 0 25	11 90	5 30	+ 5 50
	10	14 00	6 25	+ 0 25	12 60	5 61	+ 5 50
B coli	1	17 85	7 96	0 00	11 55	5 16	+ 1 75
	3	19 95	8 90	0 00	12 60	5 61	+ 2 50
	6	25 20	11 25	0 00	12 60	5 61	+ 2 75
	8	25 90	11 55	- 0 50	11 90	5 30	+ 2 50
	10	29 40	13 10	- 0 75	13 30	5 94	+ 2 75
B proteus 2	1	15 40	6 90	0 00	12 60	5 61	+ 2 25
	3	30 10	13 85	- 0 50	13 30	5 94	+ 2 25
	5	41 30	18 40	- 0 75	13 30	5 94	+ 2 25
	7	61 60	27 50	- 1 50	15 05	6 70	+ 2 25
B mesentericus b	1	30 10	13 85	0 00	11 55	5 15	+ 1 25
	3	63 00	28 10	- 2 25	11 90	5 30	+ 1 75
	5	73 50	32 80	- 2 75	13 30	5 94	+ 1 75
	7	67 20	30 00	- 2 75	15 40	6 90	+ 2 00
B mesentericus, a	1	22 60	11 85	0 00	11 90	5 30	+ 1 75
	3	68 60	30 60	- 2 50	13 65	6 10	+ 1 75
	5	72 10	32 20	- 2 75	14 00	6 25	+ 1 75
	7	67 20	30 00	- 2 75	15 05	6 70	+ 2 00

This brings to light a previously unobserved feature of bacterial metabolism, namely, the ability of certain bacteria to appropriate certain definite constituents of the protein molecule, leaving the remainder but little acted upon, at least, until the more desirable portion is largely exhausted

*Micrococcus aureus*, the Shiga bacillus and *Streptococcus pyogenes* appear to have a specific affinity for the carbohydrate radical of the protein molecule (Witte's peptone) in the absence of dextrose or of other utilizable sugar not forming an integral part of the protein molecule

## STUDIES IN BACTERIAL METABOLISM IV

By ARTHUR I KENDALL, CHESTER J FARMER, EDWARD  
P BAGG, JR AND ALEXANDER A DAY

(From the Laboratories of Biological Chemistry and Preventive Medicine  
and Hygiene, Harvard Medical School )

(Received for publication, June 15, 1912 )

In the third paper of our series of studies on bacterial metabolism,<sup>1</sup> attention was specifically directed to the fact that *Micrococcus aureus*, *B dysenteriae* (variety Shiga) and *Streptococcus pyogenes* produced acid in so-called sugar-free media, while the other organisms hitherto investigated produced alkali in this medium. In that communication we outlined what appeared to be the most logical explanation of this phenomenon, namely, that these bacteria, or at least the strains of them available at that time, actually exerted a selective action upon that portion of Witte's peptone (an important constituent of the media used for this purpose) which Pick has shown to contain a carbohydrate radical. This observation is a new one, and throws additional light upon bacterial metabolism and the way in which certain micro-organisms can actually utilize the carbohydrate radical of protein, for it will be remembered that Witte's peptone is made from fibrin. In order to demonstrate that this is a general feature of organisms of this particular type, we have isolated a series of cultures including streptococci from scarlet fever, from the vagina, from septicemia and from cellulitis, the pneumococcus and a new strain of *Mic aureus*. These coccal forms have been studied with this particular object in view. In addition to the study of reaction and ammonia production in sugar-free broth, we have followed the change in reaction and rate of ammonia formation in dextrose broth as well. The rate of fermentation of dextrose in dextrose broth has also been followed as an additional check upon our results.

<sup>1</sup> This *Journal*, xii, p 215, 1912



The tables show that all of the streptococci studied, except 34, which scarcely grew in the medium free from dextrose, produced at least some acid in the sugar-free medium. The increase

TABLE 1

*Broth F*

	DAYS	PLAIN BROTH			DEXTROSE BROTH			
		Free NH <sub>3</sub> as milligrams N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc $\frac{N}{1}$ acid per 100 cc	Free NH <sub>3</sub> as milligrams N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc $\frac{N}{1}$ acid per 100 cc	Dextrose con- sumed
Control		34 30	per cent 16 35	+0 25	34 30	per cent 16 35	+0 25	per cent
Pneumococcus	1	31 50	15 00	+0 25	31 85	15 15	+4 50	23 30
	3	35 00	16 65	+0 25	31 50	15 00	+5 25	41 00
	6	35 35	16 85	+0 50	32 90	15 65	+5 25	33 50
	8	37 80	18 00	+0 25	32 20	15 35	+5 75	52 00
Streptococcus 23	1	32 90	15 65	+0 00	32 90	15 65	+5 00	23 30
	3	32 25	15 35	+0 75	31 50	15 00	+7 75	50 70
	6	34 85	16 60	+0 25	31 85	15 15	+8 75	67 80
	8	47 60	22 65	+0 50	33 60	16 00	+9 25	76 80
Streptococcus 2	1	32 90	15 65	+0 75	31 50	15 00	+4 00	28 80
	3	32 55	15 50	+0 50	32 55	15 50	+6 50	55 50
	6	36 05	17 15	+0 75	33 60	16 00	+8 75	63 00
	8	37 80	18 00	+0 75	35 70	17 00	+8 75	66 50
Streptococcus B	1	32 20	15 30	+0 25	32 20	15 30	+1 75	23 30
	3	36 75	17 50	+0 25	34 85	16 60	+2 25	28 80
	7	42 00	20 00	+0 50	36 40	17 30	+2 25	43 80
	9	40 60	19 35	+0 25	37 10	17 65	+2 00	47 30
Streptococcus 34	1	31 85	15 15	$\pm$ 0 00	30 80	14 65	+5 25	45 20
	3	32 90	15 65	+0 25	31 50	15 00	+7 50	61 70
	7	36 05	17 15	$\pm$ 0 00	32 90	15 65	+8 25	72 00
	9	38 85	18 50	$\pm$ 0 00	33 60	16 00	+9 00	73 30
Micrococcus Aureus	1	32 90	15 65	+0 75	31 50	15 00	+3 00	23 30
	3	35 00	16 65	+0 50	32 20	15 30	+4 25	45 20
	7	42 70	20 30	$\pm$ 0 00	34 30	16 30	+4 25	56 10
	9	52 50	25 00	-0 25	36 05	17 15	+4 75	60 20

in acidity, although slight in some strains, was perfectly definite, and agrees essentially with the observation made upon the streptococcus mentioned in our previous communication <sup>2</sup>

*Mic aureus* shows correspondingly a greater acidity in plain broth, followed by a decided alkalinity, again confirming our previous observations with this organism

The very unusual amount of acid developed by *Streptococcus* 34 and 23, amounting to 9 cc of normal acid per 100 cc of media, is noteworthy Both of these organisms break down about 75 per cent of the dextrose originally present in the medium in nine days

The sparing action of dextrose, although very slight (due to the presence of a carbohydrate radical in the peptone used in the sugar-free medium, which protects the remainder of the protein from bacterial attack), is nevertheless distinct The growth curve of the streptococci is very similar to that of the other pathogenic organisms discussed previously The negative ammonia phase, mentioned in our first studies, is also clearly shown, particularly during the first days of growth

<sup>2</sup> *Loc cit*



# ON THE INFLUENCE OF GLUTARIC ACID ON PHLORHIZIN GLYCOSURIA

By A I RINGER

(From the Department of Physiological Chemistry of the University of  
Pennsylvania )

(Received for publication, June 19, 1912 )

In a series of experiments by Baer and Blum<sup>1</sup> the authors present evidence to the effect that the subcutaneous injection of glutaric acid has the power of greatly reducing the amount of sugar and nitrogen in the urine, and also to greatly diminish or cause the entire disappearance of the acetone and  $\beta$ -oxybutyric acid from the urine in phlorhizin glycosuria. In their experiment XIII, on giving 7 grams of glutaric acid, the nitrogen was reduced from 6.6 grams to 2.7 grams, the sugar from 19.5 to 2.5 grams, the acetone from 0.5299 to 0.0118 gram, the  $\beta$ -oxybutyric acid from 1.948 grams to zero. In experiment XV, the nitrogen was reduced from 8.51 to 0.49 gram in twenty-four hours, the sugar from 20.5 to 1.0 gram, acetone from 0.4312 to 0.1176 gram, and  $\beta$ -oxybutyric acid from 4.316 grams to 0.074 gram.

The authors also make the observation "je starker die Zuckerausscheidung und je schwerer die Stoffwechselstörung, die sich in Acidose kundgibt, um so ausgesprochener die Wirkung der Saure ist. Bei starker Acidose und hoher Zuckerausscheidung nach grossen Phlorhizindosen volliger Schwund des Zuckers und der Oxybuttersäure bei starkem Absinken der Stickstoffausscheidung."

The authors further express the belief that the glutaric acid has the power of preventing the formation of sugar, and consequently its elimination, by preventing the conversion of non-carbohydrate bodies into glucose. Since they state (p 100),

<sup>1</sup> Baer and Blum *Beitr z chem Physiol u Path*, x, p 80, 1907

"Für eine Zuckerbildung aus Fett im tierischen Organismus liegen überzeugende Tatsachen zurzeit nicht vor," it is evident that they believe that the glutaric acid prevents the conversion of amino-acids into glucose

With the above experiments as a foundation, the authors performed many others which were published in subsequent papers<sup>2</sup> in which they draw far reaching conclusions with regard to the metabolism of substances chemically related to glutaric acid

Baer and Blum used animals that had been phlorhizinized by single injections of phlorhizin per day In the experiments cited above, 1.5 grams of phlorhizin were injected per day On different occasions, Lusk and his pupils<sup>3</sup> have shown how little reliability can be placed in experiments on phlorhizinized dogs which are not completely under the influence of the drug The truth of this is shown very plainly in the experiments of Baer and Blum, in which they failed to get any increase in the sugar elimination after feeding glycocoll, alanine, lactic acid and glutamic acid, all substances which have been shown beyond any doubt to be converted into glucose<sup>4</sup>

With this in mind, and at Professor Lusk's kind suggestion, experiments were undertaken to study the influence of glutaric acid on completely phlorhizinized animals

**METHODS** The animals were allowed to fast for two days prior to the commencement of and throughout the experiment The animals were kept in suitable metabolism cages The urine was separated in twelve or twenty-four-hour periods (as indicated in the tables) by catheterization Merck's phlorhizin was used and was administered in 2 gram doses, three times a day, dissolved in 25 cc of 1.5 per cent  $\text{Na}_2\text{CO}_3$  solution The glutaric acid was prepared by Kahlbaum The nitrogen was determined by the Kjeldahl-Gunning method, sugar by Allihn, ammonia by Folin, acetone by Huppert-Messinger,  $\beta$ -oxybutyric acid by Magnus-Levy and total acidity by Folin's method

<sup>2</sup> Baer and Blum *Beitr z chem Physiol u Path*, xi, p 102, 1908, *Arch f exp Path u Pharm*, lxx, p 1, 1911

<sup>3</sup> For the latest reviews of the literature, see Lusk Phlorhizinglykosurie, *Ergeb d Physiol*, xii, 1912

<sup>4</sup> Mandel and Lusk *Amer Journ of Physiol*, xvi, p 129, 1916, Ringer and Lusk *Zeitschr f physiol Chem*, lxxvi, p 106, 1910

The results of the experiments are as follows

*Dog 5 Twenty-four-hour periods*

DATE	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D N	REMARKS
December 1911						
13	VI	12 7	18 0	60 9	3 38	Ten grams of glutaric acid neutralized with NaHCO <sub>3</sub> given subcutaneously in three doses during course of day
14	VII	12 2	21 1	70 3	3 33	
15	VIII	11 9	19 7	66 2	3 36	

*Dog 7 Twenty-four-hour periods*

January 1912						
18	I	13 87	16 9	63 0	3 73	Ten grams glutaric acid as above
19	II		17 9	65 5	3 66	
20	Dog in good condition Experiment discontinued for external reasons					

*Dog 22 Twelve-hour periods*

DATE	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D N	NH <sub>4</sub> -N	ACETONE AND ACETOACETIC ACID	$\beta$ OXYBUTYRIC ACID	TOTAL ACIDITY CO N NaOH 10	REMARKS
May 1912										
18	II		5 8	21 7	3 76	0 36	0 28	2 4	320 0	Ten grams glutaric acid as above
19	III	12 9	6 7	23 7	3 54	0 35	0 30	3 2	384 0	
19	IV		6 5	22 5	3 46	0 39	0 29	2 1	348 0	

These three experiments show conclusively that glutaric acid has not the influence attributed to it by Baer and Blum In none

of the experiments was there any diminution in any of the urinary constituents, in fact, a slight increase can be seen on glutaric acid days. In dog 22 there is a distinct increase in the  $\beta$ -oxybutyric acid after the administration of glutaric acid.

How can the differences of our results and those of Baer and Blum be explained? There are two possible answers. It may have been due to the presence of some impurity in their glutaric acid which affected the general metabolism to a very considerable extent and may thus account for the marked disturbance in the nitrogen elimination (0.49 gram per twenty-four hours), or it may have been due to the inadequacy of their methods as already described. It is very likely that the same misleading factors which operated in obscuring the effects of glycocoll, alanine, lactic acid, etc., operated in their glutaric acid experiments.

#### SUMMARY

Glutaric acid is shown to have no inhibitory effect on the action of phlorhizin. It does not reduce the sugar or nitrogen elimination in phlorhizin glycosuria. It does not diminish the acetone bodies in the urine.

Attention is called to the inaccuracy of the methods employed by Baer and Blum and to the misleading results.

## AUTOLYSIS OF MOLD CULTURES

By ARTHUR W. DOX AND LEONARD MAYNARD

*(From the Chemical Section of the Iowa Agricultural Experiment Station)*

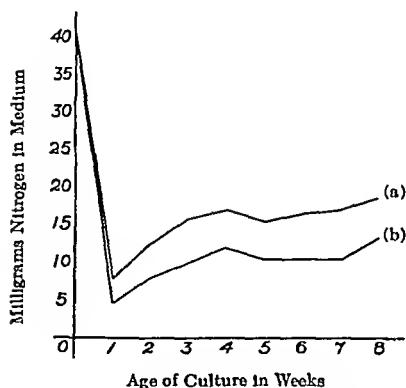
(Received for publication, June 19, 1912)

When the common saprophytic molds are grown on suitable fluid culture media, a dense turgid mycelium usually results in the course of one or two weeks. If now the culture is left undisturbed for several weeks longer the turgidity of the cells gradually disappears until finally the whole mycelium can be disintegrated by simply shaking the flask. This change in cell structure is evidently due to autolysis, and must therefore be accompanied by chemical changes which alter the composition of the substratum. Of these changes, that which the protein undergoes may be considered as perhaps the most typical and the most easily demonstrated.

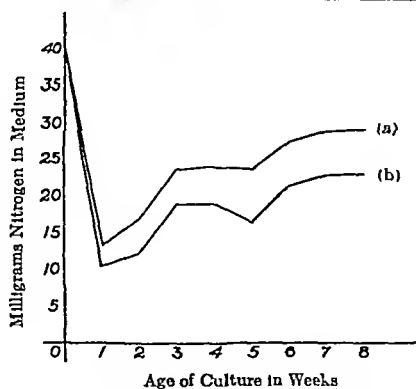
A series of experiments was undertaken in order to determine in what way and to what extent these autolytic changes affected the nitrogen content of the culture medium. It will be noted that the experiments herein described differ from those usually carried on to illustrate autolysis, in that the term is more commonly applied to the changes observed in plant or animal tissues whose vitality has been destroyed by some antiseptic.

In our experiments no antiseptic was used. In either case the essential change taking place is the digestion of the tissue by enzymes that survive the death of the cell. Where the autolysis occurs in the presence of living cells, however, there is a possibility also that the autolytic products may be accompanied by excretory products. In our own experiments it seems unlikely that such was the case to any considerable extent. In all probability metabolic activity ceases soon after the organism has exhausted the carbohydrate from the medium and the culture is alive only in the sense that it contains spores capable of germinating under proper conditions.



FIG 1 *PENICILLIUM EXPANSUM*

AGE OF CULTURE	<i>PENICILLIUM EXPANSUM</i>		<i>ASPERGILLUS NIGER</i>	
	Total N in medium	Ammonia N in medium	Total N in medium	Ammonia N in medium
weeks	mgm	mgm	mgm	mgm
0	40.7	40.7	40.7	40.7
1	7.9	4.9	13.2	10.4
2	12.3	8.0	16.8	12.1
3	15.7	10.0	23.6	18.8
4	16.8	11.8	23.9	18.8
5	15.2	10.4	23.6	16.3
6	16.3	10.4	27.5	21.3
7	16.8	10.5	28.8	22.8
8	18.5	13.2	29.2	23.0

FIG 2 *ASPERGILLUS NIGER*

The cultures used in these experiments were grown on a medium consisting of sucrose and salts, the nitrogen being entirely in the form of ammonium acid tartrate. In the first experiment a number of 200 cc Erlenmeyer flasks of uniform bottom, each containing 50 cc of the medium, were sterilized and heavily inoculated with spores. The flasks were set away in a dark place of a fairly uniform temperature. At the end of each week the cultural characteristics were noted and the contents of one flask taken for analysis. The culture fluid was filtered and the mycelium washed with distilled water. The filtrate was then diluted to a definite volume and aliquot portions analyzed for total nitrogen by the Kjeldahl method and for ammonia by distillation with magnesium oxide.

These results are more clearly illustrated by the preceding figures (1 and 2). The curves designated (a) represent the total nitrogen in the medium, those designated (b) the ammonia nitrogen.

In figure 2 it will be noticed that the nitrogen in the medium does not reach as low a minimum as in figure 1. Evidently the actual minimum is attained some time between the first and second determinations, and is therefore not indicated on the curve. The validity of this explanation is shown by the following data, represented graphically in figure 3. In this experiment the organism was grown in 300 cc flasks on 100 cc of a medium containing half the amount of nitrogen. The minimum for ammonia here represents more nearly the actual minimum.

AGE OF CULTURE	ASPERGILLUS NIGER	
	Total N in medium	Ammonia N in medium
<i>weeks</i>	<i>mgm</i>	<i>mgm</i>
0	37.5	37.5
1	7.0	2.3
2		1.8
3	12.0	5.2
4	21.0	10.2
5	22.1	15.1
6	27.4	15.4
7	24.4	17.2
8	26.6	17.8
9	27.4	19.3
26	27.8	18.5

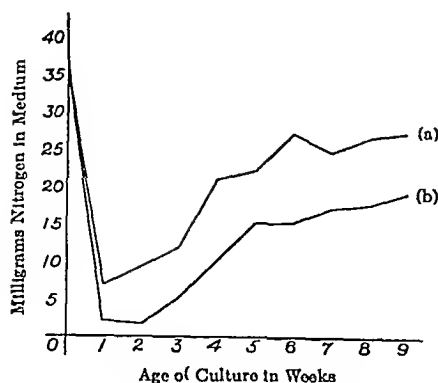


FIG 3 ASPERGILLUS NIGER

When the nitrogen in the medium is present in the form of nitrate, the mold grows equally well and shows the same cultural characteristics. The nitrogen nearly all disappears from the medium, then reappears in the form of ammonia and other soluble substances. In the following experiment, *Aspergillus niger* and *Penicillium camemberti* were grown

upon 100 cc of medium containing potassium nitrate as the source of nitrogen. The following tables indicate that the nitrate radical soon disappears and is then replaced by ammonia and other nitrogenous substances, the nature of which has not yet been determined. Indeed it would appear that the nitrate is first converted into some other form of nitrogen before assimilation takes place.

AGE OF CULTURE	ASPERGILLUS NIGER			PENICILLIUM CAMEMBERTI		
	Total N in medium	Ammonia N in medium	Nitrate N in medium	Total N in medium	Ammonia N in medium	Nitrate N in medium
weeks	mgm	mgm	mgm	mgm	mgm	mgm
0	31.6	2.1	30.0	31.6	2.1	30.0
1	10.6	1.7	2.4	19.7	1.4	2.4
2	10.6	2.0	none	10.9	2.0	none
3	8.4	2.1	none	9.2	2.2	none
4	17.4	9.5	none	15.4	7.8	none
5	27.3	19.9	none	21.0	10.2	none

These experiments all indicate that during the first week or two the mold assimilates most of the nitrogen and subsequently restores a large part of this nitrogen to the medium. In examining the curves, the fact must be taken into consideration that each determination represents an individual culture. Irregularities in the curve may therefore be due to indeterminate cultural varia-

tions Thus the dip in the curves in figure 2 at the end of the fifth week and in figure 3 at the end of the seventh week should not be interpreted as a departure from the general trend of the entire curve In seven or eight weeks an equilibrium is established after which the nitrogen in the medium appears to remain constant In the second experiment with *Aspergillus niger*, the determinations made at the end of the twenty-sixth week agreed very closely with those made at the end of the eighth week This organism restored about three-fourths of the nitrogen, which it had assimilated, to the medium, principally in the form of ammonia The remainder of the nitrogen which is still retained by the mycelium, represents nitrogen present in the spores and probably also in some chitin-like substance or glucosamine complex which does not undergo autolytic change

Determinations of total nitrogen including both medium and mycelium at the beginning and end of the experiments showed that no nitrogen had been assimilated from the atmosphere It should be mentioned also that the volatile base liberated by magnesium oxide was prepared from a culture of *Aspergillus niger* six weeks old and identified as ammonia by means of the double salt with platonic chloride

Pt	Calculated for	Found
	$(\text{NH}_4)_2 \text{PtCl}_6$	
	43.90	43.42



# A COMPARISON OF PARANUCLEIN SPLIT FROM CASEIN WITH A SYNTHETIC PARANUCLEIN, BASED ON IMMUNITY REACTIONS

By FREDERICK P GAY AND T BRAILSFORD ROBERTSON

(From the Hearst Laboratory of Pathology and Bacteriology, and the Rudolph Spreckels Physiological Laboratory, University of California )

(Received for publication, June 25, 1912 )

It has been shown by one of us (Robertson)<sup>1</sup> that a substance closely resembling paranuclein A<sup>2</sup> both in its properties and its C, H and N content can be formed from the filtered products of the complete peptic hydrolysis of an approximately 4 per cent neutral solution of potassium caseinate by the action of pure pepsin (Grubler's pepsin puriss sicc) at 36°C. In the publications referred to above, evidence has been brought forward to show that in this case we have a real synthesis of protein from the products of its hydrolytic cleavage. The solution of the products of the peptic digestion of casein which was employed in the following experiments was prepared as follows

To 6 liters of  $\frac{N}{10}$  sodium or potassium caseinate were added 2 grams of Grubler's pepsin puriss sicc which had been previously dissolved in a little water, this solution, after thorough mixing, was allowed to stand at 36° for ten days, 2 more grams of pepsin being added after the first four days (in the presence of toluol) and was then sterilized by steam at 100° and filtered through hardened filter-paper. To the filtrate were then added 2 more grams of pepsin, dissolved, as in the previous cases, in a little water, toluol introduced, and the solution was again allowed to stand at 36° for from seven to eight days, it was then again sterilized by steam at 100° and filtered through hardened filter-paper. The filtrate thus obtained is of a clear yellow color with little or no opalescence and gives no trace of a precipitate

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<sup>1</sup> T Brailsford Robertson this *Journal*, iii, p 95, 1907, v, p 493, 1909, viii, p 287, 1910, Robertson and Biddle *ibid*, ix, p 295, 1911

<sup>2</sup> Paranuclein A is formed from paranuclein of casein by the splitting off of phosphoric acid in alkaline solution (cf Salkowski and Hahn *Arch f d ges Physiol*, lxx, p 225, 1895, T Brailsford Robertson this *Journal*, iii, p 95, 1907

nor of opalescence upon the addition of acetic acid either before or after neutralization with alkali, hence both casein and paranuclein are completely absent from the solution

The synthetic paranuclein A was prepared from this solution of the products of peptic digestion in the following manner

Eighteen hundred cubic centimeters of the filtered products of the complete peptic hydrolysis of a 4 per cent solution of casein in  $\frac{N}{10}$  sodium hydrate, free from substances precipitable by acetic acid either with or without previous addition of alkali, were evaporated to 300 cc. To this solution were added 130 cc. of 10 per cent pepsin (Grübler's puriss. sicc.) and the mixture was set aside at 36° in the presence of excess of toluol. Within twenty-four hours a precipitate had appeared in the fluid. After five days the precipitate was collected on a filter and washed with water until the washings were colorless. About 200 cc. of water containing 30 cc. of  $\frac{N}{10}$  sodium hydrate were then poured into the filter and the contents agitated while the drippings were caught in about an equal volume of water containing 75 cc. of  $\frac{N}{10}$  acetic acid. The resultant precipitate was collected on a filter, washed with water, 2 liters of 99.8 per cent alcohol and 1 liter of ether (*u N d*) and dried at 36° over calcium chloride and then at room temperature over sulphuric acid. The substance thus obtained was a friable white powder very faintly tinged with yellow.

Elsewhere we show<sup>3</sup> that pure casein or paranuclein will sensitize guinea pigs for anaphylactic intoxication by either of these substances and apparently indiscriminately. Rabbits immunized by repeated injections of casein produce a potent antiserum with marked precipitins and fixation antibodies for casein and, to a somewhat less extent, for paranuclein. A much less potent antiserum was produced by paranuclein with no precipitins for either casein or paranuclein but with distinct fixation antibodies for paranuclein only. Certain of these experiments are herewith summarized and compared with others that were carried through synchronously with synthetic paranuclein A and with the products of peptic digestion.

#### ANAPHYLAXIS EXPERIMENTS

Guinea pigs were sensitized by subcutaneous injections of solutions of peptic digestion products, of synthetic paranuclein A and of paranuclein. The two latter products were dissolved in physiological saline containing 3.6 cc. of  $\frac{N}{10}$  KOH to 100 cc. The

<sup>3</sup>F. P. Gay and T. Brailsford Robertson *Journ. of Exp. Med.*, in press

animals were subsequently intoxicated by intravenous injection of one or other of the substances mentioned (table 1)

TABLE 1

GUINEA PIG	SENSITIZING DOSE	INTER VAL	SECOND DOSE (INTRAVENOUSLY)	RESULTS
114	1 cc 3 per cent paranuclein	days 28	1 cc 1 per cent paranuclein	Severe symptoms; chloroformed 2 hours, hemorrhages into peritoneum
126	Control		1 cc 1 per cent synthetic paranuclein	No symptoms, no lesions
112	1 cc 3 per cent paranuclein	28	1 cc 1 per cent synthetic paranuclein	Dead 45 minutes, immobilization of lungs, extensive hemorrhages in cecum and lungs
113	1 cc 3 per cent paranuclein	28	15 cc 1 per cent synthetic paranuclein	Severe symptoms, hemorrhages in cecum
115	1 cc 3 per cent paranuclein	28	1 cc 6 per cent solution peptic digestion	Moderate prostration, no lesions
127	Control		1 cc 6 per cent solution peptic digestion	Slight but distinct symptoms
120	Synthetic paranuclein A, 3 per cent, 1 cc	28	1 cc 1 per cent paranuclein	Severe symptoms for 2 hours No lesions
118	Synthetic paranuclein A, 3 per cent, 1 cc	28	1 cc 1 per cent synthetic paranuclein A	Severe symptoms for 2 hours Hemorrhages in stomach
122	1 cc 3 per cent solution peptic digestion	28	1 cc 1 per cent paranuclein	No symptoms, no lesions
121	1 cc 3 per cent solution peptic digestion	28	1 cc 1 per cent synthetic paranuclein A	No symptoms, no lesions
123	1 cc 3 per cent solution peptic digestion	28	1 cc 6 per cent solution peptic digestion	Slight agitation, no lesions



These experiments are unequivocal. The products of the peptic digestion of casein have distinct intoxicating effects for normal animals, a property which is already well recognized in its relation to anaphylactic shock (Vaughan and Wheeler, Biedl and Kraus). They have, however, no antigenic property nor specific intoxicating effect for animals sensitized by themselves or by paranuclein. Paranuclein and synthetic paranuclein A, however, have specific sensitizing and intoxicating properties and seem to react interchangeably. In other words, the action of pepsin on the peptic digestion solution has produced a new product that is indistinguishable from paranuclein by the anaphylaxis reaction. It would seem a true synthesized protein as claimed by Robertson.

Experiments with a potent anticasein serum corroborate and extend these biological proofs. A strong antiserum to casein which gives a precipitin reaction with this substance in dilution of 1-10,000 of a 1 per cent solution and a fixation reaction with 1-100,000 was shown to produce almost equally strong reactions with paranuclein. A fixation reaction was tried with various dilutions of the solution of peptic digestion of casein, of synthetic paranuclein A and of paranuclein in conjunction with this serum. The uniform technical details of such an experiment follow.

*Antigenic mixtures* One per cent solutions with a total volume of 1 cc

*Antiserum* 0.3 cc (56°C)

*Alexin* 0.1 cc of mixed serum from several guinea pigs removed from the clot eighteen to twenty-four hours after bleeding. Dilution to 1 cc in salt solution. Incubation with antigen and antibody at 37° C for one hour.

*Hemolytic system* 1 cc of a 5 per cent suspension of washed sheep's blood containing four minimal hemolytic doses of a strong rabbit-anti-sheep serum.

*Positive fixation* means complete absence of hemolysis after two hours at 37°, followed by sedimentation over night in the ice box.

*Controls* Each antigenic dilution plus 0.3 cc of inactivated normal rabbit serum. Antiserum with salt solution replacing antigen. These should hemolyze completely.

The results may be tabulated as follows:

*Fixation reactions with anticasein serum No 101*

Paranuclein, 1 per cent solution, Positive 0.001 cc (limit not reached)

Synthetic paranuclein A, 1 per cent solution, Positive 0.001 cc

Peptic digestion products, 1 per cent solution, Negative 0.1 cc

## SUMMARY

Paranuclein and synthetic paranuclein A (Robertson) derived from the products of complete peptic digestion of casein and synthesized by the action of pepsin at 36°C are interchangeable as tested by reactions of anaphylaxis and of alexin fixation with an anticasein serum. They have identical and specific antigenic properties that are not present in the original peptic digestion product.



# ON PHOSPHOTUNGSTIC-PHOSPHOMOLYBDIC COMPOUNDS AS COLOR REAGENTS

By OTTO FOLIN AND W DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston )

(Received for publication, June 29, 1912 )

In a recent preliminary communication Fohn and Macallum showed that it is possible to make colorimetric uric acid determinations by means of the blue color which uric acid produces with a reagent which was supposed to be phosphotungstic acid. As the supply of reagent gave out and more was prepared by boiling sodium tungstate with phosphoric acid the products obtained either failed to give the reaction or gave it so faintly as to be useless for quantitative work.

Both phosphomolybdic and phosphotungstic acids have been used as qualitative reagents for uric acid<sup>1</sup> but no one, so far as we have been able to learn from the literature, has made more than a few casual qualitative observations on the subject or has made any effort to differentiate between phosphotungstic and phosphomolybdic acids. The latter has been used more than the former probably because phosphotungstic acid as ordinarily prepared is very unsatisfactory even for qualitative purposes.

As the original reagent gave a fine reaction not only with uric acid but also with a great many phenol derivatives, including tyrosine, it seemed to us important to discover if possible how to procure a reliable supply of this interesting substance. The research has yielded some rather unexpected results.

By means of a systematic series of combinations of phosphoric acid with sodium tungstate, with sodium molybdate and with various mixtures of the two, and by assaying the chromophoric

<sup>1</sup> Offer *Centralbl f Physiol*, vii, p 801, 1894, Riegler *Maly's Jahresbericht*, 1901, p 98, 1902, p 118, Moreigne *Ibid*, 1905, p 83, Cervello *Arch f exp Path u Pharm*, lvi, p 434, 1909

value of each preparation both with uric acid and with phenol derivatives, we have obtained first, a highly sensitive reagent for uric acid which does not react with monohydric phenols or their derivatives, such as tyrosine, and secondly, a preparation which is probably far more delicate than any color reagent yet known for phenol groups. The first is a phosphotungstic acid, the second is a phosphotungstic-phosphomolybdic compound. Both are doubtless very complex compounds and we have made no attempt to isolate them in pure condition. The preparation of the reagents in solution is, however, a simple matter and that is after all for us the important point.

1 *The uric acid reagent* This solution contains 10 per cent of sodium tungstate and 16 per cent of phosphoric acid boiled together for about two hours. To 750 grams of water add 100 grams of the tungstate and 80 cc of 85 per cent phosphoric acid ( $\text{H}_3\text{PO}_4$ ). Boil gently for two hours using a reflux condenser to prevent undue concentration, cool and dilute to 1 liter. Two cubic centimeters of this solution gives the maximum color obtainable with 1 mgm of uric acid.

2 *The phenol reagent* This is a solution containing 10 per cent of sodium tungstate, 2 per cent of phosphomolybdic acid and 10 per cent of phosphoric acid. To 750 grams of water add 100 grams of sodium tungstate, 20 grams of phosphomolybdic acid and 50 cc of phosphoric acid (85 per cent). Boil for two hours with a reflux condenser, cool and dilute to 1 liter. Two cubic centimeters will give the maximum color with 1 mgm of tyrosine or uric acid.

The only precaution necessary in the preparation of these reagents is to use products which are free from nitrates. Nitric acid interferes with the color reaction. In the preparation of the uric acid reagent the tungstate must of course contain no molybdate. In the use of these reagents it is to be noted that the active compound is quickly destroyed by alkalis, that the color is produced (with uric acid and phenols) only in alkaline solutions, yet is not obtained unless the alkali is added last. From these facts we conclude that the active compound is probably reduced by the uric acid or phenol derivatives in acid solution and that the reduced compound gives blue salts on adding the alkali. The blue color obtained is also not very stable in an excess of alkali, it gradually

fades and the stronger the alkali the quicker is the fading. The selection of the right alkali is therefore important, especially in quantitative work where we want the maximum as well as the most stable color. The best alkali for the purpose is sodium carbonate. Potassium carbonate or ammonia cannot be used because they give precipitates with the reagents. The reaction is made as follows: 1 or 2 cc. of the reagent is mixed in a test tube with about the same volume of a solution containing the substance to be tested. An excess of saturated sodium carbonate solution (3-10 cc.) is then added producing the color at once. When only the most minute traces are involved solid powdered sodium carbonate is substituted for the saturated solution so as to avoid unnecessary dilution.

When made in this way, making use of solid sodium carbonate, the test is unmistakably positive with solutions containing 1 part of uric acid in 500,000 parts of water and with 1 part of tyrosine in 1,000,000 parts of water.

In order to obtain information as to the utility of the two reagents we have tried them with a large number of organic substances. Aliphatic compounds, such as acids, oxy-acids, amino-acids, carbohydrates, ketones, aldehydes and amines do not give the reactions. The same is true of indol and its derivatives. In tables I, II and III are given a number of results obtained with phenol derivatives.<sup>2</sup>

It is apparent that the uric acid reagent does not give a reaction with ordinary monohydric phenols except with those containing an amino group in the benzene ring, while it does react with di- and with poly-hydric phenols. Our phenol reagent on the other hand reacts with all oxybenzol compounds. We believe that the phenol reagent can advantageously be used as a substitute for Millon's reagent in a great many cases, particularly in the study of protein materials, because it is far more sensitive to tyrosine than Millon's (see the next paper) and the reaction comes at once and in the cold.

Through the early investigations of Salkowski and of Baumann it is known that the urine of man and animals contains a number

<sup>2</sup> For many of these compounds we are indebted to the Department of Chemistry in Harvard College.

TABLE I  
*Monohydric phenols*

NAME AND FORMULA OF SUBSTANCE TESTED	URIC ACID REAGENT	PHENOL REAGENT	MILLON'S REAGENT
Phenol, $C_6H_5OH$	—	+	+
Cresols, <i>o</i> , <i>m</i> , <i>p</i> , $C_6H_4CH_3OH$	—	+	+
Nitrophenols, <i>o</i> , <i>m</i> , <i>p</i> , $C_6H_4NO_2OH$	—	—	+
<i>p</i> -Amino-phenol, $C_6H_4NH_2OH$	+	+	+
Diethyl <i>p</i> -amino-phenol, $C_6H_2(C_2H_5)_2NH_2OH$	+	+	+
Tribromophenol, $C_6H_2Br_3OH$	—	+—	—
Thymol, $C_6H_2CH_3OHCH(CH_3)_2$	—	+	—
Carvacrol, $C_6H_2CH_3OHCH(CH_3)_2$	—	+	+
Salicylic acid, $C_6H_4OHCOOH$	—	+	+
Tyrosine, $C_6H_4OHCH_2CH(NH_2)COOH$	—	+	+

TABLE II  
*Dihydric phenols*

NAME AND FORMULA OF SUBSTANCE TESTED	URIC ACID REAGENT	PHENOL REAGENT	MILLON'S REAGENT
Hydroquinone, $C_6H_4(OH)_2$	+	+	+
Resorcin, $C_6H_4(OH)_2$	+	+	+
Pyrocatechin, $C_6H_4(OH)_2$	+	+	+
Trichlorhydroquinone, $C_6H_4Cl_3(OH)_2$	+	+	—
Orcin, $C_6H_3CH_3(OH)_2$	+	+	+
Guaiacol, $C_6H_3OCH_3OH$	+	+	+
Resorcin- <i>m</i> -methyl ether, $C_6H_3OCH_3OH$	+	+	+
Eugenol, $C_6H_3OHOCH_3CH_2CH_3$	+	+	+
Vanillin, $C_6H_3OHOCH_3CHO$	—	+	+

TABLE III  
*Trihydric phenols and other compounds*

NAME AND FORMULA OF SUBSTANCE TESTED	URIC ACID REAGENT	PHENOL REAGENT	MILLON'S REAGENT
Pyrogallol, $C_6H_3(OH)_3$	+	+	+
Phloroglucin, $C_6H_3(OH)_3$	+	+	+
Tannic acid, $C_6H_3(OH)_3$	+	+	+
Adrenalin, $C_6H_3(OH)_2CH(OH)CH_2NHCH_3$	+	+	+
Benzaldehyde, $C_6H_5CHO$	—	+	—
Morphine	+	+	—
$\alpha$ -Naphthol, $C_{10}H_7OH$	+	+	+
Naphthylamine, $C_{10}H_7NH_2$	—	+	—
Uric acid	+	+	—

of phenol derivatives but since that time there have been no systematic investigations of the subject. By qualitative tests with the above reagents we have satisfied ourselves that human urine contains polyphenol compounds even after several days' fasting. More extensive investigations concerning the quantitative relationship of the different classes of phenols and concerning their significance as metabolism products will be pursued in this laboratory.





# TYROSINE IN PROTEINS AS DETERMINED BY A NEW COLORIMETRIC METHOD

By OTTO FOLIN AND W DENIS

(From the Biochemical Laboratory of the Harvard Medical School, Boston )

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The quantitative isolation of any one amino-acid from among the heterogeneous mixtures obtained in the hydrolysis of protein materials is beset with so many practical difficulties that any chemical reaction which is specific for any amino-acid and suitable for its quantitative estimation is apt to be valuable and merit investigation. In the preceding paper we described the preparation of a new reagent which in the absence of other phenol derivatives, is specific for tyrosine. Tyrosine is one of the least soluble amino-acids, its quantitative determination should therefore be one of the most accurate, yet as a matter of fact the tyrosine contents of protein materials reported in the literature differ so much as to clearly show that the determination of this substance in amino-acid mixtures by way of its isolation in pure condition is at best a tedious and difficult task.

In illustration of the above statement it may not be out of place to cite a few examples of the discrepancies in the results of the tyrosine determinations reported by several of the most eminent workers in the field. The amount of tyrosine in zein prepared from maize is reported by Kutscher<sup>1</sup> as 10.06 per cent and by Osborne and Clapp<sup>2</sup> as 3.6 per cent. In vitelline from the hen's egg Abderhalden and Hunter<sup>3</sup> report 1.6 per cent, while Osborne and Jones<sup>4</sup> obtained 3.37 per cent tyrosine. Other examples, too numerous to mention, are to be found throughout the literature.

<sup>1</sup> *Zeitschr f physiol Chem*, xxxviii, p 111, 1903

<sup>2</sup> *Amer Journ of Physiol*, xx, p 477, 1908

<sup>3</sup> *Zeitschr f physiol Chem*, xlviii, p 505, 1906

<sup>4</sup> *Amer Journ of Physiol*, xxiv, p 153 1909

Partly because of the large discrepancies in the analyses reported in the literature, partly because we hoped to be able to determine whether proteins contain other phenol derivatives than tyrosine, it seemed to us worth while to make "tyrosine" determinations by means of our new reagent in a few definite proteins

The method used for this colorimetric determination of tyrosine in proteins is as follows One gram of the dry protein is accurately weighed out and transferred to a 500 cc Kjeldahl flask, 25 cc of 20 per cent hydrochloric acid is then added, the flask closed by means of a Hopkins condenser made from a large test tube,<sup>5</sup> and the contents of the flask boiled for twelve hours over a micro-burner At the end of this time the flame is removed, the contents of the flask transferred on cooling to a 100 cc volumetric flask and made up to volume One or two cubic centimeters of this solution are then transferred to a 100 cc volumetric flask, 5 cc of the tyrosine reagent<sup>6</sup> added, and after five minutes 25 cc of a saturated solution of sodium carbonate, and the mixture then made up to volume with cold tap water The maximum color develops in about ten minutes Therefore the reading should not be made before this time has elapsed Fading is very slow in the presence of the large excess of reagent used As nearly at the same time as possible a standard is prepared by treating 1 mgm of pure tyrosine with 5 cc of the phosphotungstic-phosphomolybdic reagent, then adding 25 cc of saturated sodium carbonate solution and making up to volume The color comparison is made by means of a Duboscq colorimeter, the standard solution being placed at 20 mm As a standard solution, we use a solution of pure tyrosine in decinormal hydrochloric acid which is made of such a concentration that 5 cc contain 1 mgm of tyrosine

In making the color comparisons both solutions should of course be absolutely clear and with no trace of precipitate, if any cloudiness is observed the solution should be filtered before being read

In the following table are given the results obtained on a number of proteins For comparison we have also compiled a list of the

<sup>5</sup> By means of this device any error due to products extracted from cork or rubber stoppers is excluded For an illustration of the apparatus, see Folin and Flanders *This Journal*, xi, p 360, 1912

<sup>6</sup> For data regarding the method of preparation of the reagent and its properties, see the preceding paper



PROTEIN	TYROSINE		ACCORDING TO
	By colorimetric method	By gravimetric method	
	<i>per cent</i>	<i>per cent</i>	
Globulin (flax seed)	3.3		
Glycinin (soy bean)	4.0	1.86	Osborne and Clapp <i>Amer Journ of Physiol</i> , xix, p 468, 1907
Phaseolin (white bean)	4.5	2.2	Osborne and Clapp <i>Ibid</i> , xviii, p 295, 1907
Globulin (squash seed)	4.9	1.4	Osborne and Clapp <i>Ibid</i> , xix, p 475, 1907
Corylin (hazel nut)	4.0		
Edestin (hemp seed)	5.2	2.1	Abderhalden <i>Zeitschr f physiol Chem</i> , xxxvii, p 499, 1903
Amandin (almond)	4.7	1.12	Osborne and Clapp <i>Amer Journ of Physiol</i> , xx, 470, 1908
Globulin (cotton seed)	4.7	2.3	Abderhalden and Ros- toski <i>Zeitschr f physiol Chem</i> , xlv, p 265, 1905
Globulin (castor bean)	4.3		
Vignin (cow pea)	4.6	2.26	Osborne and Heyl <i>Amer Journ of Physiol</i> , xxi, p 262, 1908
Legumin (pea)	4.5	2.8	Abderhalden and Bab- kin <i>Zeitschr f phys- iol Chem</i> , xlvii, p 391, 1906
Glutelin (maize)	6.5		
Zein (maize)	5.5	3.6	Osborne and Clapp <i>Amer Journ of Phys- iol</i> , xx, p 477, 1908
Hordein (barley)	4.7	1.67	Osborne and Clapp <i>Ibid</i> , xix, p 117, 1907
Gliadin (wheat)	3.3	2.4	Abderhalden and Sam- uely <i>Zeitschr f physiol Chem</i> , xlv, p 276, 1905
Glutenin (wheat)	5.8	4.25	Osborne and Clapp <i>Amer Journ of Phys- iol</i> , xvii, p 231, 1906

A glance at the above table shows that the tyrosine percentages given by our colorimetric method are in every case greater than those reported in the literature

This may be due to a number of causes (1) Our reagent may react with known amino-acids other than tyrosine (2) It may react with some amino-acid which has not yet been isolated from protein hydrolysis products (3) Phenols may be formed by the hydrolysis of proteins which would react with the reagent (4) The universally used method of determining the amount of tyrosine in proteins by the direct isolation of the amino-acid may give results much too low, due either to the fact that the substance, although insoluble, cannot be quantitatively isolated from the complex mixture of amino-acids in which it occurs, or that it may in part remain in some polypeptid combination much more soluble than the free acid, thus giving tyrosine percentages considerably lower than those obtained by the colorimetric method, which latter, as will be shown later, accounts for tyrosine held in peptid combination

In regard to the first suggestion, it may be said that we have tested our reagent with a great number of amino-acids occurring in proteins and have not found any one, other than tyrosine, which gives any reaction Oxyproline we were unable to test directly as none of the substance was available, but as gelatin, which is reported<sup>7</sup> to contain 3 per cent of this amino-acid, gives a very faint reaction with our reagent we consider it safe to assert that none of the color produced in our determinations is due to oxyproline

For lack of material we have also been unable to make any tests with oxytryptophane

In regard to the second supposition, we can offer no direct evidence, in view of the results obtained in the recent critical work on the ester method by Osborne, Abderhalden and their collaborators, it would, however, seem unlikely that appreciable quantities of any unknown amino-acids exist in the protein molecule

Our third supposition concerning the existence of phenols in the mixtures resulting from the acid hydrolysis of proteins, can, we think, be disproved by the results of the following experiment Five grams of Kahlbaum's casein were boiled for twelve hours with

<sup>7</sup> Fisher and Skita *Zeitschr f physiol Chem*, xxxv, p 221, 1902

100 cc of 20 per cent hydrochloric acid, after concentrating to a volume of about 25 cc, the solution was repeatedly shaken out with ether and the combined ethereal extracts extracted in turn with 2 per cent sodium hydrate solution. This alkaline extract when acidified and tested qualitatively with our reagent gave absolutely no trace of blue color, showing the entire absence of phenols. The same experiment was repeated using various immiscible solvents, such as chloroform, benzol, etc., in place of ether but invariably with negative results.

A similar solution of the products resulting from the acid hydrolysis of casein was also made alkaline with sodium hydrate and extracted with the above solvents on the supposition that reducing substances of a basic nature might be present, but here again negative results were obtained.

In order to test our theory that tyrosine values obtained by the usual method are invariably too low, due to the absolute impossibility of quantitatively separating this amino-acid from hydrolysis mixtures, we have made a tyrosine determination in wool by the method usually used for this purpose and have followed up the process at every stage by the use of our colorimetric reagent.

Five hundred grams of fat-free sheep's wool were boiled for twenty hours with 3 liters of 25 per cent sulphuric acid in a flask provided with a reflux condenser. At the end of this time the solution was cooled, made up to volume (5 liters) and a colorimetric tyrosine determination made. Thirty grams of tyrosine were found to be present. The solution was then treated with barium hydrate until no further precipitate was obtained with this reagent, it was then filtered and the residue repeatedly boiled with water and filtered until the filtrate no longer gave a test with Millon's reagent. At this point it still gave, however, a strong test with our phosphotungstic-phosphomolybdic reagent, and the extraction was therefore continued until only a very faint reaction could be obtained with the latter reagent.

The various filtrates were then combined, evaporated down and made up to a volume of 4000 cc. A colorimetric tyrosine determination in this solution showed the presence of 29.6 grams of this amino-acid. The solution was then evaporated down to a volume of about 2500 cc and the solid material separating out on cooling was filtered off by suction and repeatedly washed with cold water. The mother liquor was again concentrated and another crop of crystals obtained, this being repeated until four lots of crystals had been separated out. This material was then boiled with bone black and after several recrystallizations was dried at 110°, its weight was found to be 17.8 grams. When assayed by the method described above it was found to contain only 67.9 per cent tyrosine. Further purification was not attempted.

as it was not intended by this work to make a quantitative determination of tyrosine by the gravimetric method. The various mother liquors were combined, made up to volume and assayed. They were found to contain 15 grams of tyrosine.

The above experiment, we think, strongly favors our view that the discrepancies between our colorimetric tyrosine determinations and the values for this substance which appear in the literature are due to the fact that it is practically impossible to separate tyrosine quantitatively from the hydrolysis products of any protein.

The latter idea has been expressed by Osborne both in a private communication and in several publications.<sup>8</sup>

That our reagent gives the same color values with tyrosine in peptid combinations as with the same amino-acid in the free condition is proved by the fact that the length of time during which the hydrolysis is continued has no influence on the subsequent colorimetric determinations. For example, 1 gram of sheep's wool boiled with 25 cc of 20 per cent hydrochloric acid for one hour showed a tyrosine content of 6.0 per cent, when portions from the same sample of wool were boiled for five, twelve and twenty hours, respectively, exactly the same tyrosine value, 6 per cent, was obtained in every case.

The new colorimetric method employed in this investigation for the determination of tyrosine possesses of course a special advantage in that the tyrosine can be determined in a very short time and in a very small amount of substance (0.1-0.2 gram).

<sup>8</sup> Osborne and Clapp *Amer Journ of Physiol*, xvii, p 246, 1906, Osborne and Guest *this Journal*, ix, p 348, 1911.





# PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

## FOURTH PAPER

### ABSORPTION FROM THE LARGE INTESTINE

By OTTO FOLIN AND W. DENIS

(From the Biochemical Laboratory of the Harvard Medical School, Boston )

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In our second metabolism paper<sup>1</sup> we showed that the ammonia found in the portal blood represents, in a large measure, the absorption of the ammonia produced by putrefactive processes in the large intestine and we suggested that probably many other products of similar origin are absorbed from the same region. That absorption of soluble products from the large intestine does occur is evidently not a matter of doubt since "rectal feeding" is a generally accepted practice among clinicians. Nevertheless it seemed to us worth while to show experimentally that the absorption from the large intestine is extensive enough to be demonstrable by means of our new methods of blood analysis.

The experiments recorded below show that while the absorption from the large intestine, as was to be expected, is not nearly so rapid as from the small intestine, it is rapid enough under the conditions selected to cause an appreciable accumulation of the absorbed products in the blood.

In all the experiments recorded below the animals were anaesthetized by means of ether administered through a tracheal cannula and by a subcutaneous injection of morphine sulphate.

Unless otherwise noted no attempt was made to wash out the large intestine before the operation as preliminary experiments led us to believe that this is difficult to accomplish in a satisfactory manner without inducing a condition conducive to shock due to prolonged exposure of the intestines. In every case therefore a large dose of castor oil was administered about twenty-four

<sup>1</sup> This *Journal*, xi, p. 161, 1912

hours before the experiment and the animal was allowed to fast until the operation, by this treatment we obtained in every case a large intestine free from all but traces of faecal material. As soon as anaesthesia was complete a sample of normal arterial blood was drawn to serve as a control. The abdomen was then opened and ligatures placed at the ileo-caecal valve and at the lower end of the rectum.

A solution of the substance whose absorption was to be observed was then introduced into the ligatured intestine by means of a large hypodermic needle and a syringe and after withdrawal of the needle the abdominal cavity was carefully and completely closed by sutures.

Samples of blood were then drawn at suitable intervals in the manner described in an earlier paper<sup>2</sup> and analyzed for non-protein nitrogen and urea by the methods described in the same. In the experiment relating to the absorption of creatinine the colorimetric determinations of creatinine in blood were made by the method described by us in a recent paper<sup>3</sup>.

#### ABSORPTION OF UREA

**EXPERIMENT 1** Cat 58, weight 1963 grams. This animal was fed with meat thirty hours before the experiment, with the last meal it received 3 grams of castor oil.

After taking a preliminary sample of blood we injected into the ligatured intestine 25 cc. of a 10 per cent urea solution.

Samples of blood were taken at intervals, as shown below.

	<i>Mil. grams</i>
Total non-protein nitrogen per 100 cc. blood from right carotid artery before injection	42
Total urea nitrogen in the same	20
Total non-protein nitrogen per 100 cc. blood from left carotid artery thirty minutes after injection	42
Total urea nitrogen in the same	20
Total non-protein nitrogen per 100 cc. blood from right femoral artery sixty minutes after injection	46
Total urea nitrogen in the same	24
Total non-protein nitrogen per 100 cc. blood from mesenteric vein of large intestine	60
Total urea nitrogen in same	37

<sup>2</sup> This *Journal*, xi, p. 527, 1912.

<sup>3</sup> *Ibid*, xii, p. 149, 1912.

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc blood from aorta one hundred and thirty minutes after injection	52
Total urea nitrogen in same	30

No attempt was made in this experiment, or in those given below relating to the absorption of amino-acids, to determine the amount of the injected substance remaining unabsorbed at the end of the experiment, as the unavoidable faecal contamination rendered such determinations untrustworthy

#### ABSORPTION OF GLYCOCOLL

EXPERIMENT 2 Cat 54, weight 2443 grams, etherized twenty-six hours after last meal which consisted of dog biscuit Three grams of castor oil had been given by mouth twenty hours before the operation The large intestine was therefore found to be practically empty It was, however, further cleaned by a stream of warm water introduced by means of a tube thrust through the ileo-caecal valve A glass tube tied in the anus allowed the injected fluid to escape freely After emptying of all liquid we introduced into the ligatured large intestine 3 grams of glycocoll dissolved in 40 cc of warm water Samples of blood were then taken at suitable intervals, as shown below

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc blood of right femoral artery before glycocoll injection	55
Total urea nitrogen in the same	34
Total non-protein nitrogen per 100 cc blood from right carotid artery thirty minutes after glycocoll injection	58
Total urea nitrogen in the same	34
Total non-protein nitrogen per 100 cc blood from left carotid artery sixty minutes after the injection	64
Total urea nitrogen in the same	34
Total non-protein nitrogen per 100 cc blood from left femoral artery ninety-six minutes after the injection	66
Total urea nitrogen in the same	38
Total non-protein nitrogen per 100 cc blood from mesenteric vein of large intestine taken 100 minutes after injection	86
Total urea nitrogen in the same	38

#### ABSORPTION OF ALANINE

EXPERIMENT 3 Cat 57, weight 2663 grams This animal had been last fed (with meat) twenty-six hours before and had received

3 grams of castor oil twenty hours before the operation 31 grams of Kahlbaum's alanine, dissolved in 30 cc of warm water, were injected into the ligatured intestine. Analysis of samples of blood taken at appropriate intervals gave the following results

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc of blood taken from the right carotid artery before the injection	38
Total urea nitrogen in the same	20
Total non-protein nitrogen per 100 cc blood taken from the left carotid artery one hour after the injection	41
Total urea nitrogen in the same	20
Total non-protein nitrogen per 100 cc blood taken from the right femoral artery two hours after the injection	44
Total urea nitrogen in the same	24
Total non-protein nitrogen per 100 cc blood taken from the abdominal aorta three hours and six minutes after the injection	48
Total urea nitrogen in the same	26

#### ABSORPTION OF CREATININE

EXPERIMENT 4 Cat 56, weight 3013 grams This animal was fed on meat twenty-six hours before, and received 3 grams of castor oil twenty hours before the operation

After anaesthetizing, the large intestine was washed out with warm water in the manner described under Experiment 2 We then introduced into the ligatured intestine 30 grams of creatinine dissolved in 35 cc of warm water

Blood samples taken at intervals gave on analysis the following results

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc blood from right carotid artery taken just before the creatinine injection	34
Total urea nitrogen in same	20
Creatinine	none
Total non-protein nitrogen per 100 cc blood taken from left carotid artery forty-five minutes after the injection	36
Total urea nitrogen in the same	20

Creatinine was present in this sample of blood but the amount was too small to be determined quantitatively

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc in blood taken from the right femoral artery ninety-two minutes after the injection	45
Creatinine nitrogen in the same (colorimetrically determined)	11

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc in blood from the mesenteric vein of the large intestine taken seventy-two minutes after the injection	52
Creatinine nitrogen in the same	19
Total non-protein nitrogen per 100 cc blood taken from the abdominal aorta one hundred and thirty-seven minutes after the injection	50
Creatinine nitrogen in the same	14

After drawing the last sample of blood the entire large intestine was excised, emptied and carefully washed, to the intestinal contents and washings we added a little acetic acid, boiled for two or three minutes, made up (after cooling) to a volume of 500 cc and determined creatinine by the Folin method in a filtered portion of the liquid 2.24 grams of creatinine were found to have been left in the intestine, therefore 0.76 gram was absorbed

#### ABSORPTION OF WITTE'S PEPTONE

EXPERIMENT 5 Cat 55, weight 3400 grams This animal was last fed on meat thirty hours before, and received 3 grams of castor oil twenty-four hours before the operation

After taking a preliminary sample of blood we injected into the ligatured intestine 8 grams of Witte's peptone dissolved in 35 cc of warm water

Analysis of samples of blood taken at intervals gave the following results

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc blood from right carotid artery before injection	30
Total urea nitrogen in the same	18
Total non-protein nitrogen per 100 cc blood taken from left carotid artery thirty minutes after peptone injection	30
Total urea nitrogen in the same	18
Total non-protein nitrogen per 100 cc blood from right femoral artery taken sixty minutes after the injection	34
Total urea nitrogen in the same	18
Total non-protein nitrogen per 100 cc blood from the left femoral artery taken ninety-five minutes after the injection	35
Total urea nitrogen	20
Total non-protein nitrogen per 100 cc blood from mesenteric vein of the large intestine taken one hundred and two minutes after the injection	38
Total urea nitrogen in the same	22



# PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

## FIFTH PAPER

### ABSORPTION FROM THE STOMACH

BY OTTO FOLIN AND HENRY LYMAN

(*From the Biochemical Laboratory of the Harvard Medical School*)

(Received for publication, June 29, 1912)

During the last few years London, together with Abderhalden and with various assistants, has published some forty papers on digestion and absorption. One of the points repeatedly emphasized in these communications is the conclusion that there is no absorption of digestion products from the stomach<sup>1</sup>. They have made no attempt to explain and in fact have taken no notice of the more or less different results reported by earlier investigators,<sup>2</sup> evidently assuming that their own results are so conclusive as to leave no room for any further difference of opinion on the subject. The methods used by Folin and Denis for demonstrating that amino-acids are absorbed unchanged from the small and from the large intestine (see the preceding paper) are equally applicable to the study of the absorption of nitrogenous products from the stomach and in this paper we wish to report some experiments which so far as we are concerned leave no room for doubt concerning the absorption of protein digestion products from the stomach.

As in the previous work reported on the subject of absorption, cats were employed throughout. In every case the animal was anaesthetized by means of ether administered through a tracheal cannula and by a subcutaneous injection of morphine sulphate.

<sup>1</sup> See particularly *Zeitschr f physiol Chem*, lxi, p 448, 1909

<sup>2</sup> See Salaskin *Ibid*, li, p 167, 1907



The technique employed in the collection of the samples of blood and in the subsequent analysis of the same was that described in a recent paper by Folm and Denis<sup>3</sup>

In all the experiments recorded below the general method of procedure has been the same. After first taking a preliminary sample of arterial blood to serve as a control, the abdomen was opened and ligatures placed at the cardiac end of the stomach and at the pylorus. The substance whose absorption was to be studied was then dissolved in a small amount of warm water and injected into the stomach by means of a large hypodermic needle and a syringe.

The abdomen was then quickly and completely closed by means of sutures and 5 cc samples of blood taken at suitable intervals from the femoral and carotid arteries. At the end of the experiment the splenic and enteric tributaries of the portal vein were ligated (the former close to the spleen) and a sample of blood taken from the portal vein which was by this means robbed of all but its gastric branches. No attempt was made to wash out the stomach before the operation. Therefore, except in the case of creatinine, no determinations were made at the end of the experiment to ascertain to what extent absorption had taken place.

#### ABSORPTION OF GLYCOCOLL

**EXPERIMENT 1** Cat 59, weight 3703 grams. This animal had received its last meal (raw meat) twenty-four hours before the operation.

After taking a preliminary sample of blood, 61 grams of Kahlbaum's glycocoll dissolved in 40 cc of warm water were injected into the ligatured stomach.

Samples of blood taken at appropriate intervals gave on analysis the following results:

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc blood from femoral artery before injection	30
Total non-protein nitrogen per 100 cc blood from carotid artery thirty minutes after injection	38
Total non-protein nitrogen per 100 cc blood from carotid artery one hundred and sixteen minutes after injection	44

<sup>3</sup> This *Journal*, xi, p 527, 1912

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc aortic blood one hundred and fifty-eight minutes after injection	51
Total non-protein nitrogen per 100 cc portal blood one hundred and seventy-three minutes after injection	58

## ABSORPTION OF ALANINE

EXPERIMENT 2 Cat 61, weight 4040 grams This animal was last fed with meat twenty-six hours before the operation After taking a preliminary sample of blood from the femoral artery we injected into the ligatured stomach 6.36 grams of Kahlbaum's alanine dissolved in 50 cc of warm water

Analysis of samples of blood taken at intervals during the next three hours gave the following results

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc femoral blood before injection	33
Total urea nitrogen in same	18
Total non-protein nitrogen per 100 cc carotid blood thirty minutes after injection	38
Total urea nitrogen in same	18
Total non-protein nitrogen per 100 cc femoral blood one hundred and twenty minutes after injection	49
Total urea nitrogen in same	20
Total non-protein nitrogen per 100 cc femoral blood one hundred and eighty minutes after injection	52
Total urea nitrogen in same	19

## ABSORPTION OF WITTE'S PEPTONE

EXPERIMENT 3 Cat 60 weight 2313 grams This animal had fasted for twenty-six hours before the experiment After taking the usual preliminary blood sample, 8 grams of Witte's peptone dissolved in 40 cc of warm water were injected into the ligatured stomach

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc femoral blood before injection	34
Total urea nitrogen in same	31
Total non-protein nitrogen per 100 cc carotid blood thirty minutes after peptone injection	38
Total urea nitrogen in same	33

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc carotid blood sixty minutes after peptone injection	37
Total urea nitrogen in same	31
Total non-protein nitrogen per 100 cc femoral blood ninety minutes after peptone injection	40
Total urea nitrogen in same	33
Total non-protein nitrogen per 100 cc portal blood one hundred and five minutes after peptone injection	49
Total urea nitrogen in same	33

#### ABSORPTION OF CREATININE

EXPERIMENT 4 Cat 61, weight 1940 grams This animal had fasted for twenty-six hours before the experiment After taking a preliminary sample of blood from the femoral artery we injected into the ligatured stomach a solution of 2.8 grams of creatinine dissolved in 50 cc of warm water From the samples of blood drawn at intervals during the next five hours the following results were obtained

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc femoral blood before injection	38
Creatinine in same	0
Total non-protein nitrogen per 100 cc carotid blood one hour after injection	38
Creatinine in same	0
Total non-protein nitrogen per 100 cc femoral blood two hours and thirty minutes after injection	38
Creatinine in same	0
Total non-protein nitrogen per 100 cc femoral blood four hours after injection	38
Creatinine in same	0
Total non-protein nitrogen per 100 cc carotid blood five hours after injection	41
Creatinine in same	trace
Total non-protein nitrogen per 100 cc portal blood five hours and ten minutes after injection	45
Creatinine in same	trace

At the end of the experiment the stomach was excised and carefully washed out, the contents and washings were combined, treated with a few cubic centimeters of acetic acid and heated to boiling After cooling the liquid was made up to a volume of 250 cc, filtered and a colorimetric creatinine determination carried out on a

portion by the Folin method 2.50 grams of creatinine were found to have been recovered

In view of the fact that it had been found that creatinine is rapidly absorbed both from the small<sup>4</sup> and from the large<sup>5</sup> intestine, the above result was a surprise to us. We therefore immediately repeated the experiment in order to make sure that the negative result obtained was not due to any idiosyncrasy on the part of the animal used.

**EXPERIMENT 5** Cat 62, weight 3040 grams. The animal had fasted for twenty-four hours before the operation. 2.8 grams of creatinine dissolved in 50 cc. of warm water were injected into the ligatured stomach. The following results were obtained:

	<i>Milligrams</i>
Total non-protein nitrogen per 100 cc. carotid blood before injection	52
Creatinine in same	0
Total non-protein nitrogen per 100 cc. femoral blood one hour and thirty minutes after injection	52
Creatinine in same	0
Total non-protein nitrogen per 100 cc. femoral blood three hours after injection	53
Creatinine in same	trace!

Outside of the fact of proving the lack of absorption of creatinine by the stomach, the two experiments given above are of interest as they serve as controls and prove that with the stomach filled with liquid, animals may be kept under anaesthesia for as long as three hours without exhibiting changes in the non-protein nitrogen fraction of the blood.

#### ABSORPTION OF UREA

**EXPERIMENT 6** Cat 63, weight 2680 grams. This animal had fasted for twenty-two hours before the operation. 1.8 grams of urea dissolved in 25 cc. of warm water were injected into the ligatured stomach.

The following results were obtained:

	<i>Milligrams</i>
Urea nitrogen injected	1000
Urea nitrogen recovered from stomach	304

<sup>4</sup> This *Journal*, xii, p. 141, 1912.

<sup>5</sup> See preceding paper.

	<i>Milligrams</i>
Hence, urea nitrogen absorbed	696
Total non-protein nitrogen per 100 cc carotid blood before injection	49
Urea nitrogen in same	27
Total non-protein nitrogen per 100 cc carotid blood one hour and thirty minutes after injection	66
Urea nitrogen in same	42
Total non-protein nitrogen per 100 cc carotid blood three hours after injection	68
Urea nitrogen in same	45
Total non-protein nitrogen per 100 cc carotid blood four hours after injection	78
Urea nitrogen in same	54
Total non-protein nitrogen per 100 cc portal blood four hours and ten minutes after injection	86
Urea nitrogen in same	64

To determine the unabsorbed urea the stomach was excised, emptied and thoroughly washed. The various washings were combined, acidified with acetic acid and heated to boiling. After cooling the liquid was made up to a volume of 500 cc and filtered. Urea was determined in this liquid by the colorimetric method.<sup>6</sup>

The results recorded in the above experiments clearly prove that nitrogenous digestion products are absorbed from the stomach. The peculiar results reported by London and his associates are not necessarily inconsistent with ours but the interpretation drawn from them is, we believe, erroneous.

<sup>6</sup> *This Journal*, xi, p 515, 1912

# ON THE ACTION OF LEUCOCYTES ON GLUCOSE

## SECOND COMMUNICATION

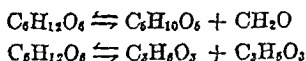
By P A LEVENE AND G M MEYER

(From the Rockefeller Institute for Medical Research, New York )

(Received for publication, July 2, 1912 )

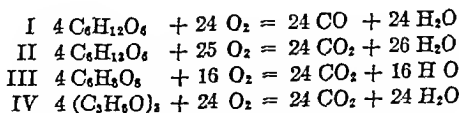
The results reported in a previous communication<sup>1</sup> were convincing of the fact that lactic acid is formed through the action of leucocytes on glucose. However, the quantity of lactic acid isolated in form of its zinc salt was lower than that of the disappeared sugar. Hence, to complete the knowledge of the action of leucocytes on glucose it was necessary to ascertain the fate of the missing sugar that had not been identified as lactic acid. The theory admits of a great variety of possibilities and an attempt was made to test by experiment some of them.

The disappearance of sugar may be caused by either one of the three reactions, oxidation, reduction or dissociation, such as



It was therefore concluded to inquire into the type of reaction which caused the disappearance of the missing sugar under the influence of leucocytes, without attempting to isolate the products of the reaction. A means for distinguishing the three types of reactions is found in the fact that the products formed in each one of them require a different quantity of oxygen for transformation into carbon dioxide.

Thus



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<sup>1</sup> This *Journal*, xi, p 361, 1912

It is seen from this that taking glucose for the unit value of oxygen requirement for oxidation into carbon dioxide, a higher value is needed for oxidation of an equal number grams molecule of the reduction products, a lower value when oxidation was responsible for the disappearance of sugar and an equal value when the breaking down of sugar was caused by a reaction of dissociation

Recently Greifenhagen, König and Scholl<sup>2</sup> have determined the conditions for oxidation of several carbohydrates, of methyl alcohol, and of glycerin into carbon dioxide by means of a potassium permanganate solution. The authors recommend the method for quantitative estimation of sugar

It was concluded to resort to this method for estimation of the oxygen requirement for transformation into carbon dioxide of the products formed by the action of leucocytes on glucose. However, before applying it, the method was tested in regard to the following requirements

1 Whether it permits of detecting accurately such small differences in sugar concentration as occur in course of our leucocyte experiments

2 Whether it permits of detecting saccharic acid in such concentrations as may occur in the leucocyte experiments

The method was found to satisfy these two requirements and it was therefore applied to the analysis of the products of reaction of leucocytes on glucose

It was found that while the reducing power of the sugar solution suffered the usual loss of about 10 per cent, the oxygen consumption of the original sugar remained unaltered at the end of the experiment. A control of the leucocyte extract treated in the same manner as in the principal experiment did not demonstrate any estimable reducing power on permanganate solution. On the basis of these experiments one was forced to the conclusion that the products of the action of leucocytes on glucose are neither oxidation nor reduction products

It also was known from the results of previous experiments that the substances formed in the reaction were not volatile and did not possess reducing power for Fehling's solution

The possibilities that suggested themselves on the basis of these

<sup>2</sup> *Biochem Zeitschr*, xxxv, p 169, 1911

data were, either that all the missing glucose was transformed into lactic acid, or that part of it was transformed into glyceric aldehyde which in its turn suffered Cannizzaro's transformation into glyceric acid and glycerol. The attempts to detect glycerol in our reaction products were not successful.

These considerations seemed to point to the conclusion that all the missing sugar was transformed into lactic acid and yet only about 40 per cent of the missing sugar was extracted in form of the acid. Thus we were led once more to test the efficiency of our method of extracting lactic acid. In the previous communication mention was made of the fact that about 90 per cent of lactic acid was recovered when the acid was added to a suspension of leucocytes and the mixture was treated in the same manner as the reaction mixture of the principal experiments. In the course of the present work an experiment was made with a view of establishing the action of leucocytes on lactic acid. In that experiment the lactic acid was allowed to remain in contact with the leucocytes for thirty-six hours at  $37^{\circ}\text{C}$ , and in that instance nearly all the added lactic acid was recovered by extraction. This experiment may also serve as a test of the efficiency of estimating lactic acid by the extraction method. However, there were lacking experiments testing the efficiency of the extraction of lactic acid from mixtures containing only about 10 per cent of the acid and 90 per cent of glucose. Buchner and Meisenheimer<sup>3</sup> have made such experiments with mixtures of lactic acid and saccharose and have regained by extraction about 90 per cent of the acid present in the mixture. We have repeated their experiments and have obtained as good results as they. However, when such experiments were performed with mixtures containing glucose instead of saccharose, only about 40 to 50 per cent of the lactic acid present in it were extracted—approximately the same values as those obtained in our principal experiments. This is due perhaps to the fact that the physical nature of the glucose mixture does not permit of a very intimate mixing with the sodium sulphate powder and that, in its turn, makes the extraction of lactic acid imperfect. Thus the extraction method can serve only to identify the lactic acid formed in course of the experiment and cannot be relied upon for its quanti-

<sup>3</sup> *Ber d deutsch chem Gesellsch*, xliii, p 1784, 1910



tative determination For this, use has to be made of the oxidation method by means of permanganate solution A test experiment showed that the values obtained by the oxidation method are identical with those obtained by titration On the basis of these considerations we feel justified in the conclusion that lactic acid is the only product of the action of leucocytes on glucose

### CONCLUSIONS

1 Under the influence of leucocytes glucose undergoes dissociation into lactic acid only

2 The lactic acid formed under these conditions remains intact

3 There is no evidence of the formation of any oxidation products of glucose in the same experiments

4 It is remarkable that under conditions approaching those existing in the animal body the transformation of glucose is so simple and uniform while the formation of lactic acid from glucose by purely chemical methods is always accompanied by numerous side reactions

### EXPERIMENTAL PART

The technique of the experiments was the same as in the preceding communication The details of permanganate titration method are as follows

The protein-free sugar solution was made up to a definite volume and a quantity containing approximately 30 mgm of sugar used for each oxidation This was accomplished by boiling with 40 cc of  $\frac{N}{5}$  permanganate solution and 60 cc of 10 per cent potassium hydrate in a round bottom, long necked, 500 cc Jena flask, fitted with a long wide tube to prevent loss of liquid by possible bumping Bumping was kept at a minimum by adding a sufficient quantity of glass pearls and boiling vigorously over a wire gauze Boiling was continued for ten minutes

To the hot solution were then added 50 cc of 25 per cent sulphuric acid and sufficient  $\frac{N}{5}$  oxalic acid solution to completely decolorize the solution which was titrated back to pink with  $\frac{N}{10}$  permanganate solution The amount of permanganate used for the oxidation of the sugar is the difference between the total  $\frac{N}{5}$  permanganate solution (cc  $\frac{N}{10}$  permanganate reduced to cc  $\frac{N}{5}$ ) less the number cc  $\frac{N}{5}$  oxalic acid added The determination takes about twenty minutes

*Total oxidation of sugar by method of Greifenhagen, showing differences of 1 mgm sugar*

Different volumes of a 2 per cent solution of glucose were oxidized by the process described earlier in this paper. The results are presented in the following table. All reported values represent averages of several determinations.

GLUCOSE SOLUTION	GLUCOSE	$\frac{N}{5}$ $\text{KMnO}_4$	$\frac{N}{5}$ OXALIC ACID	DIFFERENCES	DIFFERENCES FOR 1 MOM
cc.	mgm	cc	cc	cc	cc
15 0	30	40 99	21 03	19 96	
15 5	31	42 48	22 10	20 38	0 42
16 0	32	43 82	22 75	21 07	0 68
16 5	33	43 88	22 10	21 78	0 71
17 0	34	44 18	21 70	22 48	0 70
					Average
					0 6275

Calculated difference for 1 mgm glucose = 0 666 cc

*Oxidation of saccharic acid*

Acid potassium saccharate was prepared in the laboratory by the usual process. The crude salt was recrystallized several times before it was used for the experiment. It was dried to constant weight in a vacuum desiccator over phosphorus pentoxide. 0 496 gram of the salt was dissolved in 100 cc of water. Of this solution 10 cc were employed for oxidation with 40 cc of  $\frac{N}{5}$  permanganate solution and 60 cc of a 10 per cent solution of potassium hydroxide. For decoloration it was necessary to add 23 5 cc of  $\frac{N}{5}$  oxalic acid. The colorless solution was titrated to permanent pink color by 2 0 cc of  $\frac{N}{5}$  permanganate solution, it required 18 5 cc of  $\frac{N}{10}$  permanganate solution to oxidize 0 0496 gram of acid potassium saccharate.

The equivalent amount of glucose requires for oxidation 24 9 cc of permanganate.

*Oxidation of mixtures of saccharic acid and glucose*

For this series of experiments two solutions were prepared (A) 30 mgm glucose dissolved in 250 cc of water and (B) 41 mgm acid potassium saccharate dissolved in 250 cc of water. Twenty-

five cubic centimeters of each of these solutions were oxidized separately according to the method previously described. A mixture of these solutions consisting of 23.30 cc of the glucose solution and 1.64 cc of the acid potassium saccharate solution was also subjected to this process. The fractional parts of a cubic centimeter were measured off on a specially graduated pipette.

The following table summarizes the results

SOLUTION	GLUCOSE (A) OR SAC- CHARATE (B)	$\frac{N}{5}$ $\text{KMnO}_4$	10 PERCENT KOH	ADDI- TIONAL $\frac{N}{5}$ $\text{KMnO}_4$	$\frac{N}{5}$ OXALIC ACID	DIFFER- ENCE $\frac{N}{5}$ $\text{KMnO}_4$
cc	mgm	cc	cc	cc	cc	cc
25.00 (A)	30.0	40	60	2.9	22.5	20.6
25.00 (B)	41.0	40	60	0.7	25.5	15.2
23.30 (A)	28.0	40	60	3.8	24.1	19.7
1.64 (B)	2.2					

#### *Oxidation of leucocytes and phosphate solution alone*

For this series of experiments there was prepared a mixture consisting of a leucocyte suspension and of a Henderson phosphate solution combined in the same proportions as employed in the glycolysis experiments. The mixture was allowed to stand at 37°C for thirty-six hours.

The proteins were then removed by coagulation and subsequent filtration. Twenty-five cubic centimeters of the filtrate were boiled with 40 cc  $\frac{N}{5}$  permanganate solution and 60 cc of a 10 per cent solution of potassium hydroxide. For decoloration were employed 45.0 cc of  $\frac{N}{5}$  oxalic acid. The excess of oxalic acid was oxidized by 6 cc of  $\frac{N}{5}$  permanganate solution. Hence 1 cc of  $\frac{N}{5}$  permanganate solution was required to oxidize 25 cc of the tested solution. Considering that the 25 cc of the filtrate used is five times the quantity used in the sugar oxidations, the possible error in those determinations due to organic matter in the leucocytes would be equal to 0.2 cc  $\frac{N}{5}$  permanganate solution, which amount is insignificant.

#### *Action of leucocytes on glucose*

In this series of experiments the reduction values for Fehling's solution were compared with those for a permanganate solution. The comparative estimation was made at the beginning and at

the end of the experiments A suspension of sterile leucocytes in a Henderson phosphate solution containing sugar was allowed to stand thirty-six hours at 37°C The reduction of the Fehling's solution was measured by Volhard's method and of the permanganate solution by the process described earlier in the paper

The reducing power of the solution was also tested after the glycolyzed solution had been boiled for two hours with a 2 per cent hydrochloric acid solution This experiment aimed to ascertain whether or not condensation was responsible—perhaps in part—for the disappearance of glucose

#### Sugar determination

	ORIGINAL SOLUTION USED	NH <sub>4</sub> CNS	NH <sub>4</sub> CNS PER CC	GLUCOSE	LOSS
	cc	cc	cc	per cent	per cent
Before	1	22 5	22 5	8 05	
After 36 hrs	1	21 2	21 2	7 58	5 82

#### Oxidation

	ORIGINAL SOLUTION USED	N $\frac{5}{3}$ KMnO <sub>4</sub>	ADDI- TIONAL N $\frac{5}{3}$ KMnO <sub>4</sub>	N $\frac{5}{3}$ OXALIC ACID	DIFFER- ENCE	SUGAR	LOSS
	cc	cc	cc	cc	cc	per cent	per cent
Before	0 5	40	9 15	22 70	26 45	7 92	
After 36 hrs	0 5	40	6 37	20 00	26 39	7 90	0 02

#### Sugar reduction

	ORIGINAL SOLUTION USED	NH <sub>4</sub> CNS	NH <sub>4</sub> CNS PER CC	GLUCOSE	LOSS
	cc	cc	cc	per cent	per cent
Before	2	26 00	13 00	4 65	
After 36 hrs	2	23 40	11 70	4 18	10 10

#### Oxidation

	ORIGINAL SOLUTION USED	N $\frac{5}{3}$ KMnO <sub>4</sub>	ADDI- TIONAL N $\frac{5}{3}$ KMnO <sub>4</sub>	TOTAL N $\frac{5}{3}$ KMnO <sub>4</sub>	N $\frac{5}{3}$ OXALIC ACID	N $\frac{5}{3}$ KMnO <sub>4</sub> UTILIZED	GLUCOSE
	cc	cc	cc	cc	cc	cc	per cent
Before	0 5	45 00	4 20	47 10	32 00	15 10	4 50
After 36 hrs	0 5	45 00	0 60	45 30	30 50	14 80	4 46

*Sugar reduction*

	ORIGINAL SOLUTION USED	NH <sub>4</sub> CNS	NH <sub>4</sub> CNS PER CC	GLUCOSE	LOSS
	cc.	cc	cc	per cent	per cent
Before	2	26 10	13 05	4 67	
After 36 hrs	2	22 80	11 40	4 07	15 00
Hydrolysed	2	23 20	11 60	4 15	

*Oxidation*

	ORIGINAL SOLUTION USED	$\frac{N}{5}$ KMnO <sub>4</sub>	ADDI- TIONAL* $\frac{N}{5}$ KMnO <sub>4</sub>	$\frac{N}{5}$ OXALIC ACID	DIFFER- ENCE	SUGAR
	cc	cc	cc	cc	cc	per cent
Before	1	40 00	4 70	13 70	31 00	4 65
After 36 hrs	1	40 00	2 60	9 50	31 10	4 68

\*  $\frac{N}{10}$  permanganate solution was used in titration against  $\frac{N}{5}$  oxalic acid. In this and in the subsequent tables the values are all recalculated as  $\frac{N}{5}$  permanganate solution.

*Oxidation of lactic acid*

For these experiments there was prepared an approximately  $\frac{N}{5}$  solution of lactic acid. The lactic acid content of the solution was determined according to F. Ulzer and H. Seidel.<sup>4</sup> To 10 cc of the solution an excess of  $\frac{N}{10}$  sodium hydroxide solution was added, using phenolphthalein as indicator. The mixture was brought to a boil and titrated back with  $\frac{N}{10}$  hydrochloric acid solution. It was found that 1 cc of the lactic acid solution contained 0.016 gram lactic acid. For the experiments the original solution of lactic acid was diluted with nine volumes of water. Two cubic centimeters of the diluted solution were employed for each oxidation experiment. The solution was boiled with 40 cc of  $\frac{N}{5}$  potassium permanganate and 60 cc of 10 per cent potassium hydroxide. For decoloration 22.2 cc of  $\frac{N}{5}$  oxalic acid were used. In order to oxidize the excess of oxalic acid it was required to add 37 cc of  $\frac{N}{5}$  potassium permanganate solution. Hence in order to oxidize 20 cc of lactic acid solution there were employed 21.5 cc of  $\frac{N}{5}$  potassium permanganate. As 1 cc of  $\frac{N}{5}$  potassium permanganate oxidizes 1.5 mgm lactic acid, the 2 cc of the original solution oxidized contained 0.0322 gram of lactic acid. The value obtained by titration was 0.0320 gram.

<sup>4</sup> *Monatsh d. Chem.*, xviii, pp 130-141

*Action of leucocytes on lactic acid*

A leucocyte suspension from 700 cc of exudate was mixed with Henderson's phosphate solution containing 0.470 gram of lactic acid, as determined by titration. One-half of the lactic acid mixture was analyzed immediately for lactic acid, the other portion after remaining at 37° for thirty-six hours. For this purpose the mixture was freed from protein by boiling and acetic acid. The clear filtrate was neutralized and evaporated to a small volume, acidified with phosphoric acid, ground up with anhydrous sodium sulphate, and extracted with ether. The zinc salt was prepared as already described.

	<i>Zinc Lactate</i>	<i>Lactic Acid</i>
By titration		0.2350
Control before	0.3158	0.2322
After 36 hours	0.3122	0.2312

*Extraction of lactic acid*

There were prepared two solutions of lactic acid. One contained glucose the other sucrose. The concentration of the sugar was 5 per cent. Five grams of glucose or sucrose were dissolved in 100 cc of water containing a definite amount of lactic acid, as determined on a separate sample by titration or by conversion to the zinc salt. The mixtures of sugar and lactic acid were evaporated to a small volume faintly acidified with phosphoric acid, ground with anhydrous sodium sulphate and extracted with anhydrous ether until portions of the extract failed to give the Uffelmann test. 0.2751 gram zinc lactate was obtained.

Leucocytes and sugar mixtures containing no lactic acid, prepared according to the manner previously described, were subjected to the same treatment. The table summarizes the results.

EXPERIMENT	GLUCOSE	LOSS	LACTIC ACID ADDED	ZN LACTATE RECOVERED	LACTIC ACID	LACTIC ACID REGAINED
	<i>per cent</i>	<i>per cent</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>per cent</i>
Leucocytes + glucose + lactic acid	5.01	10		0.2544	0.1815	36.40
Glucose	5.00		0.4725	0.2751	0.1963	41.50
	5.00		0.5000	0.3375	0.2680	53.40
Sucrose	5.00		0.4220	0.5245	0.3760	89.00



# THE QUANTITATIVE DETERMINATION OF ALIPHATIC AMINO GROUPS II

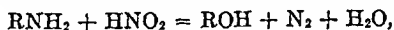
## PLATE I

By DONALD D. VAN SLYKE

(From the Rockefeller Institute for Medical Research, New York)

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The author's method for determining aliphatic amino nitrogen<sup>1</sup> by measurement of the nitrogen gas evolved in the reaction,



has proven capable of sufficiently general application<sup>2</sup> to make it

<sup>1</sup> This *Journal*, ix, p 185, 1911

<sup>2</sup> It has been utilized in the following articles Levene and Jacobs Hefenucleinsäure III, *Ber d deutsch chem Gesellsch*, xlii, p 3150, Sjollem and Rinkes Hydrolyse des Kartoffeleiweisses, *Zeitschr f physiol Chem*, lxxvi, p 506, Kossel and Cameron Die freien Amidgruppen der einfachsten Proteine, *ibid*, lxxvi, p 457, Brach and von Furth Chemische Aufbau des Chitins, *Biochem Zeitschr*, xxxviii, p 468, Medigreceanu Composition of Urinary Albumin, *Journ of Exp Med*, xiv, p 298, White Comparative Proteolysis Experiments with Trypsin, *Journ Amer Chem Soc*, xxviii, p 1911, Robinson Organic Nitrogenous Compounds in Peat Soil, *Tech Bull No 7, Michigan Agricultural Experiment Station*, Mays Proteinkörper des Liebig'schen Fleischextraktes, *Zeitschr f physiol Chem*, lxxviii, p 47, Abderhalden and Van Slyke Bestimmung des Aminostickstoffs in einigen Polypeptiden, *ibid*, lxxvi, p 506, Abderhalden and Kramm Abbau der Proteine im Darmkanal, *ibid*, lxxvii, p 425, Abderhalden and Kramm Abbau der Milcheiweisskörper durch Magensaft unter verschiedenen Bedingungen, *ibid*, lxxvii, p 463, Abderhalden and Hanslian Verwendbarkeit der Estermethode zum Nachweis von Monamino-säuren neben Polypeptiden, *ibid*, lxxvii, p 285, Abderhalden Fütterungsversuche mit vollständig abgebauten Nahrungsstoffen, *ibid*, lxxvii, p 22, Osborne and Guest Hydrolysis of Casein, this *Journal*, ix, p 333, Osborne and Guest Hydrolysis of Wheat Gliadin, *ibid*, ix, p 425, Levene, Van Slyke and Birchard Partial Hydrolysis of Proteins II and III, *ibid*, viii, p 269, x, p 57, Van Slyke and White Digestion of Protein in the Stomach and Intestine of the Dogfish, *ibid*, ix, p 209, Van Slyke Analysis of Proteins by Determinations of Chemical Groups, *ibid*, x, p 15, Van Slyke and Birchard Nature of the free Amino Groups in the Native Proteins, *Proc Soc Exper Biol and Med* ix 1912



appear worth while to publish certain improvements made in the apparatus during the past year. Without complicating the manipulation, increasing the cost<sup>3</sup> or sacrificing any of the accuracy of the original, the present apparatus has several advantages over the former. (1) The deaminizing vessel, *D*, need not be disconnected from the gas burette between analyses. The apparatus can be used for an indefinite number of determinations without disconnecting the parts. (2) Both the deaminizing bulb *D* and the Hempel pipette can be shaken by a motor. This is a tremendous convenience when considerable series of analyses have to be made.<sup>4</sup> (3) With the new apparatus one can work even more rapidly than with the original. *D* can be shaken more efficiently with a motor than is possible by hand and the reaction is correspondingly accelerated. Furthermore, during the last stage of the analysis, while the nitric oxide is being removed in the modified Hempel pipette, *D* may be charged with fresh nitrous acid and freed from air (first stage of next analysis), so that one can run off determinations at the rate of one every seven or eight minutes. (4) The motor increases not only the convenience and speed of the method but also its reliability. The only errors likely to occur in the original method were those due to insufficient shaking of *D* or of the pipette, in the former case the nitrogen is incompletely expelled from the nitrous acid solution, and results are too low, in the latter case the nitric oxide is incompletely absorbed and results are too high. The possibility of the occurrence of either error is reduced to a minimum by a mechanical shaking device. (5) The use of rubber tubes to carry off overflows and used up solutions decidedly enhances the neatness of the apparatus, facilitates cleaning, and protects the hands of the manipulator from nitrous acid.

The first of the above cited advantages, freedom from the necessity of disconnecting the apparatus between analyses, was

<sup>3</sup> Robert Goetze, 4 Hortelstrasse, Leipsic, supplies the glass parts for about Mk. 28, Emil Greiner, 45 Cliff Street, New York, for \$10.

<sup>4</sup> When the method is used for infrequent analyses, the motor is, of course, superfluous. Even when the shaking is done by hand, however, the new apparatus is more convenient than the old. The parts, minus the driving mechanism, are arranged as shown in the photograph, except that the modified Hempel pipette is brought around to the side of the gas burette opposite the deaminizing bulb where it can be reached more conveniently.



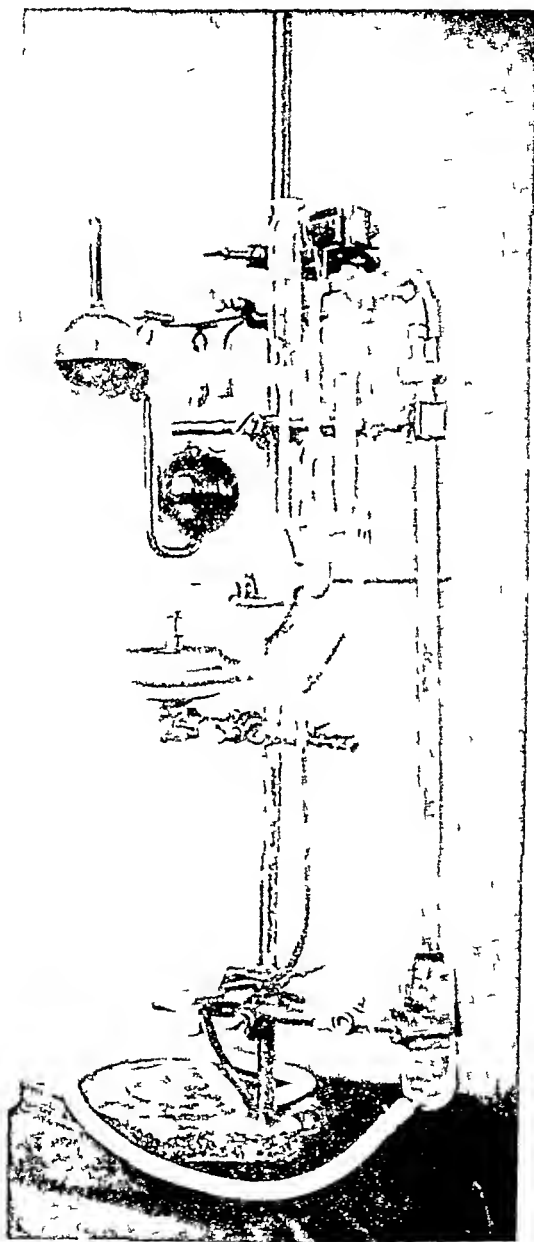


FIG 1 ENTIRE APPARATUS ASSEMBLED FOR PERMANENT USE

introduced by Klein<sup>5</sup> who substituted for the rubber-stoppered deaminizing bottle of the original apparatus, a bulb, to the bottom of which, as in *D*, the inlets and outlets for liquid were sealed, while the gas escaped through a capillary at the top. Klein's modification, compared with the original, had the disadvantages that the amino solutions could not be measured directly into the deaminizing chamber from a burette, like *B*, but had to be washed in, and that the time required for the determination was lengthened by the changes required in the manipulation. Nevertheless, the advantage of a one-piece, all-glass deaminizing vessel was made so evident by the use of Klein's modification that we attempted to combine this advantage with those of our original apparatus. The present apparatus is the result of this attempt, with the additional adaptation of both deaminizing vessel and Hempel pipette to use with a mechanical shaker.

The structure of the apparatus and the manner in which it is set up are apparent from the accompanying cut and photograph.

*D* is of 40-45 cc capacity, *A* of about 35 cc and the burette *B* of 10 cc. The wire from which the deaminizing bulb *D* is suspended should be fairly stiff, and rigidly fastened in position from above so that the loop about the capillary acts as a fixed center. *A* is then so placed that its center of gravity comes near this center and the shaking of *D* is accomplished with a minimum motion in *A* and, consequently, without putting a dangerous strain on the tube which connects *A* with *D*. This tube is strong-walled and of 3 mm inner diameter. It is essential that the bore of cock *a* should also be 3 mm. The reason for this is that during the analysis gas containing some nitrogen collects in the tube. Unless *a* is of as wide bore as the tube the liquid from *A* may flow around the bubble instead of forcing it into *D* at the end of the reaction. The cock *d* is also of large bore in order to facilitate emptying *D*. The neck connecting *D* and *B* must be of at least 8 mm inner diameter in order to allow free circulation of the solution in *D* up to the cock *B*. The small bulb at the top of *D* keeps the reacting solution from splashing into the capillary.

In order to insure tightness of the cocks and to prevent their becoming loosened by vigorous shaking it is well to lubricate them with a paste made by dissolving together over a flame one part of rubber, one part of paraffin and two parts of vaseline.

The structure of the modified Hempel pipette is entirely apparent from the photograph. This form would undoubtedly facilitate absorption in all gas analyses where shaking is necessary.

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<sup>5</sup> This *Journal*, x, p 287

cases of some native proteins which, when deaminized, form unwieldy coagula that mechanically interfere with the thorough agitation of the mixture, a longer time may be required. In case a viscous solution is being analyzed and the liquid threatens to foam over into *F*, *B* is rinsed out and a little caprylic alcohol is added through it. For amino substances, such as amino-purines, requiring a longer time than five minutes to react (cf p 191, former article), one merely mixes the reacting solutions and lets them stand the required length of time, then shakes about two minutes to drive the nitrogen completely out of solution.

When it is known that the solution to be analyzed is likely to foam violently, it is advisable to add caprylic alcohol through *B* before the amino solution. *B* is then rinsed with alcohol and dried with ether or a roll of filter paper before it receives the amino solution.

3 *Absorption of nitric oxide and measurement of nitrogen* The reaction being completed, all the gas in *D* is displaced into *F* by liquid from *A* and the mixture of nitrogen and nitric oxide is driven from *F* into the absorption pipette. The driving rod is then connected with the pipette by lifting the hook from the shoulder of *d* and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the pipette. The latter is then shaken by the motor for a minute, which, with any but almost completely exhausted permanganate solutions, completes the absorption of nitric oxide. The pure nitrogen is then measured in *F*. During the above operations *a* is left open, to permit displacement of liquid from *D* as nitric oxide forms in *D*.

*Testing completeness of reaction* Particularly when the mechanical shaker is used, there is little danger of failing to obtain a complete evolution of nitrogen. The point may be tested, however, as follows. The nitrogen from *F* is driven out at *c*, *a* is closed and *D* connected with *F*. The gas which has formed in the nitrous acid solution in *D* during the absorption of the nitric oxide and measurement of nitrogen is shaken out and driven over into *F* and then into the Hempel pipette as before. After absorption of the nitric oxide, the gas left should not measure more than that obtained in blank tests, usually less than 0.1 cc. After the gas has all been forced from *D* over into *F* at the end of the reaction, the nitrous solution is run out from *D*, by opening *d*, through a tube

leading to a drain *B* is rinsed and dried with a roll of filter paper or with alcohol and ether and the apparatus is immediately ready for use again

Blank determinations, performed as above except that 10 cc of distilled water replaces the solution of amino substance, must be performed on every fresh lot of nitrite used. The amount of gas obtained on a five-minute blank is usually 0.3 to 0.4 cc, with very little increase for longer tests. Nitrite giving a much larger correction should be rejected.

The following determinations, performed with an  $\frac{N}{10}$  solution of leucine indicate the speed of the reaction. The correction applied for reagents was 0.40 cc. Ten cubic centimeters of  $\frac{N}{10}$  leucine solution, containing 14.01 mgm of nitrogen, were used for each determination.

TIME OF REACTION	N	TEMPERATURE	PRESSURE	N OBTAINED	N OBTAINED ON SECOND SHAKING OF SOLUTION	TOTAL N OBTAINED
minutes	cc	degrees C	mm	mgm	cc	mgm
2	24.38	23	762	13.71	0.45	13.97
3	24.65	22	762	13.93	0.20	14.03
4	24.80	22	762	14.01	0.00	14.01
10	25.07	24	762	14.03	0.00	14.03

The driving wheel was making 300 revolutions per minute. At speeds of 400 or 500 revolutions the reaction can be driven to completion in three, or, with higher room temperature, in two minutes.

The rate of reaction of ammonia is shown in the following table. Ten cc portions of  $\frac{N}{5}$  ammonium sulphate solution, containing 28.02 mgm of nitrogen each, were used.

TIME OF REACTION	N	TEMPERATURE	PRESSURE	WEIGHT OF N	PER CENT OF TOTAL AMMONIA NITROGEN
minutes	cc	degrees C	mm	mgm	
3	12.1	24	752	6.86	21.6
5	18.4	24	752	10.16	36.3
10	31.5	24	752	17.38	62.1

As pointed out before, ammonia reacts slowly compared with the amino-acids. For accurate determination of  $NH_2$  nitrogen in digest-

ing solutions, etc., it is advisable to first remove the ammonia, although good comparative results can be obtained, in the presence of the relatively small proportion of ammonia usually present, if reaction conditions of time, temperature, and concentration of solutions are kept constant, so that the proportion of the ammonia decomposed is the same in each determination. The ammonia can be conveniently removed and determined by distillation with  $\text{Ca}(\text{OH})_2$  under diminished pressure, as described on page 21, vol. X of this *Journal*. After the distillation the excess  $\text{Ca}(\text{OH})_2$  is dissolved with acetic acid. It is essential that all the ethyl alcohol should be distilled off, as it decomposes nitrous acid with formation of large volumes of gases which can be removed with permanganate only with difficulty and by the use of perfectly fresh permanganate solution. The point at which the alcohol has all been boiled off is usually indicated when the solution begins to foam in the distilling flask.

The following results were obtained with lysine picrate. Lysine, as previously stated, reacts more slowly than the other amino-acids because it contains not only an  $\alpha$ -amino group but also an  $\omega$ -amino group. In the fifteen and thirty-minute determinations the solution was shaken only during the last five minutes.

WEIGHT OF LYSINE PICRATE	TIME OF REACTION	N	TEMPER- ATURE	PRESSURE	NH <sub>2</sub> -N FOUND	NH <sub>2</sub> -N CALCULATED
gram	minutes	cc	degrees C	mm	per cent	per cent
0.200	5	25.4	24	764	7.13	7.47
0.200	15	26.7	24	764	7.49	7.47
0.200	30	26.7	24	764	7.49	7.47

Solutions to be analyzed should be free of ethyl alcohol and acetone. These substances when mixed with nitrous acid give off gases or vapors which are with difficulty absorbed by the permanganate.

*Amyl alcohol, which in the original description of the amino method was recommended to prevent the foaming of viscous solution, must be replaced for this purpose by caprylic alcohol [Kahlbaum's "octyl-alkohol (sekundär) I"] Amyl alcohol, boiling at 131°, has the disadvantage of a very noticeable vapor tension. Permanganate solution apparently possesses the power to absorb slight amounts*

of amyl alcohol vapor. Particularly on hot days, however, and when relatively much of the alcohol is used, it is necessary to change the permanganate with every analysis or else reduce the volume of gas observed by multiplication with an empirically determined factor.

The following determinations illustrate this point. They were performed with  $\frac{N}{10}$  leucine<sup>7</sup> solution, using 1 cc of amyl alcohol in each case. The temperature was 27°, the pressure 756 mm. The analyses were done in rapid succession, using the same permanganate solution with all

NUMBER	$\frac{N}{10}$ LEUCINE SOLUTION	GAS OBSERVED	GAS CALCULATED FOR N	$\frac{\text{GAS OBSERVED}}{\text{GAS CALCULATED}}$
	cc	cc	cc	
1	10	26.1	25.7	1.015
2	10	27.8	25.7	1.082
3	10	27.6	25.7	1.079
4	10	27.6	25.7	1.079
5	5	13.8	13.05	1.079

Theoretically a higher alcohol should be more adapted to the purpose, since, as the size of the alcohol molecule increases, the volatility diminishes, while the effectiveness in reducing the surface tension of aqueous solutions increases. Kahlbaum's secondary octyl (caprylic) alcohol, which is not very expensive, was found satisfactory in every respect. It is so efficient in preventing foam that when a few drops are added, a 2 or 3 per cent egg albumin solution can be analyzed without difficulty, even when the reacting solution is shaken rapidly with a motor. The fact that it does not interfere to the slightest extent with the accuracy of the results is shown by the following determinations. They were performed

NUMBER	$\frac{N}{10}$ LEUCINE SOLUTION	GAS OBSERVED	GAS CALCULATED FOR N
	cc	cc	cc
1	10.00 ± 0.04	25.95	25.95 ± 0.10
2	10.00 ± 0.04	25.90	25.95 ± 0.10
3	10.00 ± 0.04	25.95	25.95 ± 0.10
4	10.00 ± 0.04	26.00	25.95 ± 0.10
5	10.00 ± 0.04	25.85	25.95 ± 0.10

<sup>7</sup> For results with glycocholic cf accompanying paper on glycocholic picrate



in the same manner as those with amyl alcohol and with the same  $\frac{N}{16}$  leucine solution. The temperature was 29°, as warm as a laboratory often becomes, and the pressure 756 mm. The first determination was a control, without the octyl alcohol.

For convenience in calculating results the following table is appended. The figures are calculated by dividing by 2 those for moist nitrogen given by Gattermann in the *Praxis des organischen Chemikers*, ninth edition. They represent the weights of amino nitrogen in milligrams which correspond to 1 cc. of nitrogen gas, obtained by the action of nitrous acid and measured over water, at the temperatures and atmospheric pressures indicated.

mm pressure

8	760	762	764	766	768	770	772	<i>t</i>
915	0 5935	0 5950	0 5965	0 5980	0 5995	0 6010	0 6030	11°
890	0 5905	0 5925	0 5940	0 5955	0 5970	0 5985	0 6000	12°
865	0 5880	0 5895	0 5910	0 5930	0 5945	0 5960	0 5975	13°
840	0 5855	0 5870	0 5885	0 5900	0 5915	0 5935	0 5950	14°
810	0 5830	0 5845	0 5860	0 5875	0 5890	0 5905	0 5920	15°
785	0 5800	0 5815	0 5830	0 5850	0 5865	0 5880	0 5895	16°
760	0 5775	0 5790	0 5805	0 5820	0 5825	0 5850	0 5865	17°
730	0 5745	0 5765	0 5780	0 5795	0 5810	0 5825	0 5840	18°
705	0 5720	0 5735	0 5750	0 5765	0 5780	0 5795	0 5810	19°
675	0 5690	0 5705	0 5725	0 5740	0 5755	0 5770	0 5785	20°
650	0 5665	0 5680	0 5695	0 5710	0 5725	0 5740	0 5755	21°
620	0 5635	0 5650	0 5665	0 5680	0 5695	0 5715	0 5730	22°
595	0 5610	0 5625	0 5640	0 5655	0 5670	0 5685	0 5700	23°
565	0 5580	0 5595	0 5610	0 5625	0 5640	0 5655	0 5670	24°
535	0 5550	0 5565	0 5580	0 5595	0 5610	0 5625	0 5640	25°
505	0 5520	0 5535	0 5550	0 5565	0 5580	0 5595	0 5610	26°
475	0 5490	0 5505	0 5520	0 5535	0 5550	0 5565	0 5580	27°
445	0 5460	0 5475	0 5490	0 5505	0 5520	0 5535	0 5550	28°
415	0 5430	0 5445	0 5460	0 5475	0 5490	0 5505	0 5520	29°
385	0 5400	0 5415	0 5430	0 5445	0 5460	0 5475	0 5490	30°
8	760	762	764	766	768	770	772	<i>t</i>



# THE COMPOSITION AND PROPERTIES OF GLYCOCOLL PICRATE AND THE SEPARATION OF GLYCOCOLL FROM ALANINE

By P A LEVENE AND DONALD D VAN SLYKE

(From the Rockefeller Institute for Medical Research, New York )

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Glycocoll picrate was first described by Levene,<sup>1</sup> who demonstrated its value in separating the mixture of glycocoll and alanine encountered in the hydrolysis of most proteins. He assigned to it the structure,  $C_2H_6NO_2 \cdot C_6H_3N_3O_7$ , regarding the substance as a combination of one molecule each of glycocoll and picric acid. We find that this was an error, and that, as reported by us some months ago at the Christmas meeting of the Society of Biological Chemists, the picrate contains *two* molecules of glycocoll in combination with one of picric acid. The fact that Levene and others who have since used the picrate in the isolation of glycocoll have not detected this error is easy to explain. Picric acid and glycocoll contain practically the same percentage of carbon and nitrogen, so that determinations of the percentages of these elements, which are ordinarily most to be relied upon, give no clue whatever to the proportions in which the two constituents of the salt are present. Even the difference in hydrogen contents is so low that picrates with respectively one and two molecules of glycocoll would vary by only 0.8 in their hydrogen percentage. Consequently the entire elementary composition affords no reliable data for determination of the structure of the substance.

In addition to this, the problem was complicated by the readiness with which free picric acid crystallizes. When enough or more than enough picric acid to form the salt of supposed monomolecular composition is added in hot solution to the glycocoll, a portion of the excess of picric acid crystallizes with the picrate when the

<sup>1</sup> This *Journal*, 1, p. 413

solution is cooled. In this manner it is easy to obtain mixtures of glycocoll picrate and free picric acid containing almost as much of the latter as is required by the monomolecular formula. The excess picric acid is, however, merely a mechanical admixture. It can be removed by recrystallizing the substance, or by merely shaking it out with ether, which leaves behind the pure salt,  $(C_2H_5NO_2)_2 C_6H_3O_7N_3$ .

For determining analytically the purity of the salt, and in particular its freedom from excess picric acid, the elementary composition is, as already stated, of little significance. Two other methods are available. (1) The proportion of glycocoll can be estimated by determination of the amino nitrogen by the nitrous acid method.<sup>2</sup> This is the simplest method, and the one by which the correct composition of the picrate was first detected. (2) The salt can be decomposed with an excess of mineral acid, the picric acid shaken out with ether and the glycocoll and picric acid weighed separately. Results by this method confirm those by the amino determination.

#### EXPERIMENTAL

##### *The reaction of glycocoll with nitrous acid*

As already stated by one of us,<sup>3</sup> glycocoll when treated with nitrous acid gives off a volume of gas, not absorbed by alkaline permanganate, which exceeds by several per cent that calculated for one molecule of nitrogen. Results are sufficiently constant, however, to enable one to obtain good figures for the amino nitrogen if an empirical correction is used. As the amino determination is the only practical method for analysis of the picrate, the following analyses of glycocoll were made in order to determine accurately the empirical correction under definite conditions.

In the analyses given below the solutions of glycocoll were mixed with the nitrous acid in the apparatus described in the previous article. The reacting solutions were allowed to stand five, eleven or thirty minutes, then shaken one minute by hand to expel the dissolved nitrogen. Glycocoll contains 18.69 per cent of nitrogen.

<sup>2</sup> Van Slyke this *Journal*, ix, p 185, also, cf preceding article

<sup>3</sup> Van Slyke this *Journal*, ix, p 199

WEIGHT OF GLYCOCOLL	TIME OF REACTION	GAS MEASURED	TEMPERATURE	PRESSURE	PER CENT N CALCULATED FROM GAS VOLUME	PER CENT OF THEORETICAL N
gram	minutes	cc	degrees C	mm		
0 0801	6	27 8	21	770	19 94	106 7
0 0801	12	28 7	21	770	20 59	110 1
0 0801	31	29 2	21	770	21 01	112 4

The following results were obtained from analyses in which the reacting solutions were shaken constantly and rapidly with a motor from the time at which they were mixed till the reaction was discontinued

WEIGHT OF GLYCOCOLL	TIME OF REACTION	GAS MEASURED	TEMPERATURE	PRESSURE	PER CENT N CALCULATED FROM GAS VOLUME	PER CENT OF THEORETICAL N
gram	minutes	cc	degrees C	mm		
0 0768	1	28 0	24	754	19 72	105 5
0 0768	2	28 6	24	754	20 13	107 7
0 0768	3	28 7	24	754	20 20	108 0
0 0768	5	28 6	24	754	20 13	107 7
0 0768	5	28 25	20	758	20 12	107 7
0 0768	40*	29 00	20	758	20 58	111 3

\*The 40-minute determination was shaken only during the last five minutes

From the above figures it is apparent that when the reacting solutions are allowed to stand five minutes and are then shaken one minute, the results require, for correction, multiplication by the factor,  $\frac{100}{106.7}$  or 0.94, while, when the mechanical shaker is used for two to five minutes, a factor of 0.93 is required. The above conditions and corrections were used in the analyses of glycocoll picrate, with consistent results. It should be noted, that while making analyses of pure glycocoll solutions one must change the permanganate absorbent solution oftener than when analyzing other amino-acids, as the abnormal gases given off by glycocoll are less completely taken up by the permanganate after the latter has been used for four or five analyses of this amino-acid.

#### *Composition of glycocoll picrate*

1 Samples of glycocoll picrate prepared according to Levene's original directions, using 4 parts of picric acid to 1 of gly-

cocoll (1.5 parts is the proportion actually required to form  $(C_2H_5NO_2)_2 \cdot C_6H_3N_3O_7$ ), showed amino nitrogen contents varying between that required for the supposed monoglycine picrate and diglycine picrate. The melting points varied between  $185^\circ$  and  $195^\circ$ . When such picrates were recrystallized from enough solvent to hold in solution the free picric acid present the pure diglycine picrate was obtained, softening at about  $200^\circ$  and decomposing at  $202^\circ$ . The following case is an example. A picrate obtained by Levene's original method from gelatin showed an amino content of 6.9 per cent and a melting point of  $188^\circ$ . It was recrystallized from water and then melted at  $200^\circ$ . It gave the following figures on analysis:

Substance, 0.1900, 25.7 cc N at 768 mm,  $21^\circ$  (with  $HNO_3$ )

Substance, 0.1541,  $CO_2$ , 0.1796,  $H_2O$ , 0.0492

	Calculated for $(C_2H_5NO_2)_2 \cdot C_6H_3N_3O_7$	Found
NH-N	7.39	7.28
C	31.67	31.76
H	3.43	3.57

Five grams of the picrate were dissolved in 50 cc of hot water and the picric acid freed with 30 cc of  $\frac{N}{2}$  sulphuric acid. The picric acid was shaken out with ether. Evaporation of the ethereal extract yielded 3.001 grams of picric acid melting at  $122^\circ$ . The sulphuric acid in the aqueous solution was precipitated by the addition of an exact equivalent of barium hydrate solution which had been titrated against the same  $\frac{N}{2}$  sulphuric acid. The filtrate from the barium sulphate was concentrated to dryness and yielded 2.007 grams of glycocoll melting at  $228-230^\circ$ .

	Calculated for $(C_2H_5NO_2)_2 \cdot C_6H_3H_3O_7$	Found
Picric acid	60.26	60.02
Glycocoll	39.74	40.15

The glycocoll gave the following figures on analysis:

Substance, 0.1240,  $CO_2$ , 0.1463,  $H_2O$ , 0.0752

	Calculated for $C_2H_5NO_2$	Found
C	31.97	32.19
H	6.73	6.84

2. Samples of glycine picrate can readily be prepared, containing abnormally high amounts of picric acid, by crystallization from solutions containing more than the required 0.5 molecule of picric acid per molecule of glycocoll. That the excess of picric acid is

merely a mechanical admixture, however, can be shown by extracting it with anhydrous ether

Two grams of glycocoll and 6 grams (1 molecule) of picric acid were dissolved in 20 cc of hot water and allowed to crystallize at 0°. The crystals were washed with ice water, followed by a little alcohol and ether and dried in a vacuum. Yield, 3.79 grams. The substance softened at 185° and melted at 190°.

ANALYSIS 0.1726 gram substance, 18.7 cc N at 20°, 764 mm. Using the factor 0.94, this gives amino nitrogen as 5.85 per cent. The calculated amount for the diglycine picrate is 7.39, for the monoglycine picrate, 4.61 per cent.

The substance was ground fine and extracted with several portions of boiling anhydrous ether, the extraction being continued until the extracts were no longer colored. The residue now obtained melted at 200° and the amino nitrogen content was raised to nearly the calculated value for the pure diglycine picrate.

ANALYSIS 0.1529 gram substance, 20.25 cc N at 18°, 760 mm

	Calculated for (C <sub>6</sub> H <sub>5</sub> NO <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>5</sub> N <sub>2</sub> O <sub>7</sub>	Found
NH <sub>2</sub> -N	7.39	7.19

As before, the factor 0.94 was used.

Another sample with high picric acid content was obtained by dissolving 7.6 grams of diglycine picrate in 20 cc of water with 4.6 grams (1 molecule) of picric acid. The product, obtained by crystallization at 0°, weighed 5.49 grams and contained only 5.49 per cent of amino nitrogen. After extraction with ether it gave figures for the pure diglycine picrate.

ANALYSIS 0.1423 gram substance, 19.5 cc N at 21°, 770 mm

	Calculated for (C <sub>6</sub> H <sub>5</sub> NO <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>5</sub> N <sub>2</sub> O <sub>7</sub>	Found
NH <sub>2</sub> -N	7.39	7.42

Incidentally these experiments bring out the fact that the presence of an excess of free picric acid greatly increases the solubility of glycine picrate. Twenty cubic centimeters of water at 0° dissolve only 0.35 gram of diglycine picrate, equivalent to 0.14 gram of glycocoll. In the presence of an excess of picric acid, however, there remained in solution in the first of the above two experiments, 0.8 gram of glycocoll, and in the second, 1.4 gram.



3 The presence of free picric acid does not prevent the crystallization of diglycine picrate in pure condition, provided the solvent used is capable of holding in solution all the excess picric acid. The latter decreases the yield of diglycine picrate, but does not affect its composition nor crystallize with it.

In each of the following three experiments 2 grams of Kahlbaum's glycocoll were dissolved in 10 cc of water. To these solutions were added respectively one, two and three times the amount of picric acid required to form diglycine picrate, the acid being in each case dissolved in 40 cc of 95 per cent alcohol before it was added to the glycocoll solution. It was previously ascertained that 50 cc of 76 per cent alcohol are capable of holding in solution, even at 0°, the amount of free picric acid present. The solutions were allowed to stand over night at 0° for crystallization to become complete. The crystals were washed in each case with uniform portions of 50 per cent alcohol, 95 per cent alcohol and once with ether. The results of the experiments are given in the following table.

OLYCOCOLL	PICRIC ACID	YIELD OF GLYCINE PICRATE	AMINO N CONTENT	NH-N CALCULATED
grams	grams	grams	per cent	per cent
2	3	4.53	7.15	7.39
2	6	2.87	7.44	7.39
2	9	1.52	7.41	7.39

The picrate melted at 199°-200° in each case.

#### *Conditions for precipitation of glycine picrate*

Glycine picrate is much less soluble in alcohol than in water, but, as alcohol precipitates the amino-acids from which glycocoll has usually to be separated, the conditions for precipitation of the latter as completely as possible in water solutions had to be determined. In the following experiments a supersaturated solution was made by dissolving glycine picrate in warm water. Portions of this solution were cooled to the temperatures indicated and maintained there for several hours. They were then filtered and 10 cc portions of the filtrates used for determinations of amino nitrogen. All the solutions deposited crystals as soon as they were cooled to their respective temperatures and they were stirred

occasionally while standing in order to assure approximate attainment of solubility equilibrium

TEMPERATURE	N	TEMPERATURE OF N	PRESSURE	GRAMS GLYCOCOLL DISSOLVED IN 100 cc	GRAMS GLYCINE PICRATE DISSOLVED IN 100 cc
degrees C	cc	degrees C	mm		
21	29.6	16	754	1.72	4.35
11	16.2	13	752	0.95	2.42
0	11.8	16	766	0.698	1.76
0	11.95	18	764	0.698	1.76

From the above it is evident that the solubility of glycine picrate rises very rapidly with the temperature and that cooling completely to 0° is necessary to attain most complete precipitation.

The following experiment was performed in order to determine approximately how much time is required for precipitation at 0° to reach its maximum. A 10 per cent solution of glycine picrate was cooled to 0° by shaking the containing flask in ice water. Crystallization began before the solution reached 0° and was complete within fifteen minutes after that time. Portions of the solution were filtered off and used for amino determinations as described in the preceding experiment.

TIME OF REACTION	N	TEMPERATURE OF N	PRESSURE	GRAMS GLYCOCOLL DISSOLVED IN 100 cc	GRAMS GLYCOCOLL PICRATE DISSOLVED IN 100 cc
	cc	degrees C	mm		
15 min	11.90	18	764	0.694	1.75
3 hrs	12.20	22	764	0.698	1.76

Gravimetric solubility determinations showed that 50 per cent alcohol at 21° dissolves per 100 cc, 2.13 grams of glycine picrate, absolute alcohol only 0.16 gram.

#### *Separation of glycocoll from alanine*

In a solution containing only glycocoll and picric acid it has been shown above that the presence of an excess of picric acid above the amount required to form diglycine picrate increases the solubility

of the latter and makes the precipitation less complete. When, however, alanine is also present it prevents the solvent effect of an excess of picric acid, at least so long as the amount of the latter does not exceed one-half molecule for each atom of amino-acid nitrogen present. In fact, the presence of excess picric acid up to this point actually depresses the solubility of the glycine picrate, and makes its precipitation more complete than when only enough picric acid to combine with the glycocoll is added. The following two experiments illustrate this point.

*Separation of alanine and glycocoll using only enough picric acid to combine with the glycocoll.* One gram of glycocoll, 1.5 grams (1 molecule) of picric acid and 1 gram of alanine were dissolved in 15 cc. of hot water and cooled to 0°. After several hours the crystals were filtered on a cold suction funnel, washed twice with a few cubic centimeters of ice water and then several times with 95 per cent alcohol. The dried product weighed 1.96 grams, containing 0.78 gram of the original 1.00 gram of glycocoll. The picrate was pure, softening first at 200° and decomposing at 202°.

*Separation of glycocoll from alanine, using one-half molecule of picric acid for each molecule of amino-acid.* One gram of glycocoll, 1 gram of alanine and 2.78 grams of picric acid were dissolved in 15 cc. of water. The precipitate was treated in the same manner as that in the preceding experiment. In this case, however, the yield was larger, 2.22 grams of picrate, equivalent to 0.89 gram of glycocoll. The melting point was 197° showing that the picrate was not entirely pure. This was confirmed by the analysis, which, however, indicates that the picrate was 96 per cent pure, containing about 4 per cent of free picric acid.

Substance, (I) 0.1550 gram, (II) 0.1680 gram, N, (I) 20.3 cc., (II) 22.2 cc. at 20°, 764 mm.

	Calculated for (C <sub>7</sub> H <sub>5</sub> NO <sub>5</sub> ) <sub>2</sub> · C <sub>3</sub> H <sub>7</sub> N <sub>2</sub> O <sub>2</sub>		Found
NH <sub>2</sub> -N	7.39	I	II
		7.05	7.11

*Separation of glycocoll from alanine, with isolation of the alanine.* The alanine used in this experiment, like that in the two preceding, was an optically active sample prepared from hydrolyzed silk and purified by repeated recrystallization. It was analytically pure and gave the following figures for rotation in the yellow rays of a light purified by a Schmidt and Haensch spectroscope.

Substance, 0.1214, solution, containing an equivalent of HCl, 1.568 gram, concentration of alanine hydrochloride, 10.91 per cent, sp gr, 1.03, rotation in 1 dm tube,  $+117^{\circ}$

$$[\alpha]_D^{25} = +10.5^{\circ} \pm 0.1^{\circ}$$

Fischer gives  $10.4^{\circ} \pm 0.2^{\circ}$  The glycocoll used was Kahlbaum's

One gram of alanine, 1 gram of glycocoll and 1.8 grams of picric acid (0.6 molecule for the glycocoll) were dissolved in 7 cc of hot water. The solution was allowed to stand at  $0^{\circ}$  until crystallization was complete. The crystals were washed as before on an ice-cold suction funnel, with a little ice water, followed by alcohol. The yield was 2.29 grams, equivalent to 0.91 gram of glycocoll. The product decomposed at  $202^{\circ}$  and was quite pure.

ANALYSIS 0.1611 gram substance, 22.9 cc N

	Calculated for ( $C_2H_5NO_2$ ) <sub>2</sub> $C_2H_5N_3O_7$	Found
NH <sub>2</sub> -N	7.39	7.40

The analysis was performed with the mechanical shaker so that the factor 0.93 was used.

The filtrate from the picrate was acidified with 20 cc of  $\frac{N}{2}$  sulphuric acid and shaken out several times with ether, until the yellow color of picric acid had entirely disappeared. The solution was then heated on the steam bath and the sulphuric acid was precipitated by the addition of an exact equivalent of barium hydrate solution which had previously been titrated against the sulphuric acid used. The filtrate from the barium sulphate was evaporated to dryness. It left 1.09 grams of residue which contained 1.0 per cent of ash. The substance on combustion gave figures approximating those for alanine, but 0.5 per cent low, indicating the presence of a small proportion of glycocoll.

Substance (ash-free), 0.1275 gram, CO<sub>2</sub>, 0.1871, H<sub>2</sub>O, 0.0890

	Calculated for $C_2H_5NO_2$	Calculated for $C_2H_5N_3O_7$	Found
C	31.97	40.42	39.92
H	6.73	7.93	7.85

The sharpest differentiation between the two substances is given by the rotation, as glycocoll is inactive. The rotation, like the yields of picrate and alanine, indicated that 9 per cent of the glycocoll had gone into the filtrate with the alanine.

Substance (ash free), 0.1120 gram, solution, containing an equivalent of HCl, 1.551 gram, concentration, of alanine hydrochloride, 10.15 per cent, sp. gr., 1.03, rotation,  $+0.99^\circ$

$$[\alpha]_D^{20} = +9.5^\circ \pm 0.1^\circ$$

We confirmed the experience of Levene in failing entirely to obtain a picrate of alanine. No compound of picric acid and alanine could be induced to crystallize from any concentration in water solution, and, when such a solution of alanine and picric acid was precipitated by addition of alcohol, the precipitate consisted of pure alanine.

#### SUMMARY

Glycine picrate is composed of two molecular weights of glycocoll in combination with one of picric acid. It softens at  $199^\circ$ – $200^\circ$  and decomposes at  $202^\circ$ . A correct melting point is ordinarily good proof of its purity, as the presence of a few per cent of free picric acid or of alanine depresses the melting point several degrees. The picrate is extremely soluble in hot water. At  $0^\circ$ , however, 100 cc. dissolve only 1.76 grams of picrate, containing 0.7 gram of glycocoll.

To separate glycocoll from alanine, the mixture is dissolved in three to four parts of hot water. In this solution is also dissolved an amount of picric acid which exceeds that required to combine with the glycocoll present (1.5 grams of picric acid combine with 1 gram of glycocoll), but does not exceed that required to combine with the mixture, if all the amino nitrogen present is calculated as belonging to the glycocoll. The solution is cooled to  $0^\circ$  and allowed to remain at that temperature until crystallization is complete, which usually occurs in less than an hour. The glycine picrate is washed with a slight amount of water at  $0^\circ$ , followed by 95 per cent alcohol. The purity is controlled by the melting point and amino nitrogen determination. The filtrate from the picrate is treated with an excess of  $\frac{7}{8}$  sulphuric acid and freed from picric acid with ether. The sulphuric acid is then precipitated by an equivalent of titrated barium hydrate solution. The alanine is left as a residue when the filtrate from the barium sulphate is concentrated to dryness. It still contains several per cent of glycocoll, but can be obtained, as above described, over 90 per cent pure.

# THE CONDITIONS FOR COMPLETE HYDROLYSIS OF PROTEINS

By DONALD D. VAN SLYKE

(From the Rockefeller Institute for Medical Research, New York.)

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Henriques and Gjaldbaek,<sup>1</sup> in an article devoted chiefly to a study of the above subject with the formol titration, find that, when proteins are hydrolyzed with acid, both amino nitrogen and ammonia increase up to a certain point, at which the amino nitrogen attains its maximum. When hydrolysis is continued beyond this point, a transformation of amino nitrogen into ammonia follows, indicating a deaminization of some amino-acid or acids. The point at which the amino nitrogen reaches a maximum, with the least possible formation of ammonia, they define as the *end point* of the hydrolysis.

From the results of acid hydrolyses performed under various conditions Henriques and Gjaldbaek conclude that the most certain method for obtaining the above end point is treatment with 3*N* hydrochloric acid in an autoclave at 150° for one and one-half hours. With casein, Witte peptone and calves' flesh, complete hydrolysis could also be obtained by boiling with 20 per cent hydrochloric acid for twelve hours. With egg albumin, however, the results were different. The amount of amino nitrogen present after six hours' boiling with 20 per cent acid was only 89 per cent of that obtained by autoclave hydrolysis, and continuation of the boiling for six hours more resulted in no increase. From this it appeared that, in egg albumin at least, some peptid linkings exist in such an unusually stable condition that they can be split only by the autoclave hydrolysis.

Both Osborne and Guest<sup>2</sup> and the writer<sup>3</sup> have based protein analyses on the assumption that hydrolysis is complete when the

<sup>1</sup> *Zeitschr f physiol Chem*, lxxvii, p 8

<sup>2</sup> *This Journal*, ix, p 335, ix, p 425

<sup>3</sup> *Ibid*, x, p 15, 1911

amino nitrogen set free by boiling with 20 per cent hydrochloric acid reaches a maximum (sometimes attained only after twenty-four hours or more boiling) The results of Henriques and Gjaldbaek with egg albumin necessitate a test of the validity of this assumption

#### EXPERIMENTAL

Portions of 1.5 grams of each protein, in air-dry condition, were placed in test tubes of 100 cc capacity To the tubes used in autoclave experiments 50 cc portions of 3*N* hydrochloric acid were added, for experiments at 100° the same volume of 20 per cent hydrochloric acid was used The tubes for autoclave hydrolysis were warmed in a water bath for a short time when necessary in order to bring the proteins completely into solution before placing them in the autoclave The latter was placed in an oil bath at 175° When the bath and autoclave reached equilibrium the temperature had fallen to 150°, and it was maintained at this point for the one and one-half or three hours duration of the experiment, at the end of which the autoclave was removed from the bath and allowed to cool in the air The tubes for hydrolysis at 100° were loosely stoppered and were immersed in a bath of boiling water for intervals of ten, twenty-four, or forty-eight hours

After hydrolysis the hydrochloric acid was distilled off as completely as possible under diminished pressure The ammonia was then determined by distillation at 15-20 mm pressure with calcium hydrate and alcohol, as described in the writer's method for analysis of proteins <sup>4</sup> The undissolved calcium hydrate was filtered off and the adsorbed melanin determined by the Kjeldahl method <sup>5</sup> The filtrate was neutralized with hydrochloric acid, concentrated under diminished pressure and brought to 100 cc Duplicate portions of 20 cc were used for Kjeldahl determinations, and 10 cc portions for estimation of amino nitrogen by the nitrous acid method <sup>6</sup> The difference between the Kjeldahl and  $\text{NH}_2$  determinations gives the non-amino ( $\text{NH}$ ) nitrogen This includes one  $\text{NH}_2$  group, that of the guanidine nucleus of arginine, which does not react with nitrous acid The results are given in percentages of the total nitrogen of the proteins

<sup>4</sup> This *Journal*, x, p 20

<sup>5</sup> *Ibid*, x, p 21

<sup>6</sup> *Ibid*, ix, p 185

TABLE I

*Casein*

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150°	150	160°
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs	1½ hrs	3 hrs	3 hrs
NH <sub>3</sub>	8.9	9.4	10.1	9.8	9.8	12.1
NH <sub>2</sub> *	65.2	72.4	72.1	72.6	71.1	67.9
NH	23.8	16.1	16.1	16.3	17.0	18.0
Melanin	2.1	2.1	1.8	1.3	2.0	2.0

\* Osborne and Guest (*This Journal* ix p 334) found 71.7 per cent of nitrogen present as NH<sub>2</sub> in casein completely hydrolyzed by boiling 48 hours with 20 per cent HCl

TABLE II

*Edestin*

TEMPERATURE OF HYDROLYSIS	100	100°	100	110**	150°	150°	160
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs		1½ hrs	3 hrs	3 hrs
NH <sub>3</sub>	8.7	9.1	9.9	10.0	9.8	12.9	15.4
NH <sub>2</sub>	53.5	61.9	62.8	61.1	60.3	61.6	59.3
NH	36.2	27.5	25.3	26.3	27.0	24.5	24.3
Melanin	1.8	1.4	2.1	1.9	2.9	1.0	1.0

\* Results at 110° are taken from figures published in the paper on Analysis of Proteins *this Journal* x p 15. The proteins were boiled with 20 per cent hydrochloric acid twenty or more hours until the amino nitrogen became constant.

TABLE III

*Wheat gliadin*

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	110 †	150°	150
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs		1½ hrs	3 hrs
NH <sub>3</sub>	23.7	24.4	24.8	25.5	24.5	25.6
NH <sub>2</sub> *	51.3	57.3	57.1	57.2	56.9	57.1
NH	22.7	16.1	16.0	16.3	16.4	17.2
Melanin	2.3	2.2	2.1	0.9	2.1	0.2

\* Osborne and Guest (*This Journal* ix p 425) obtained 59.2 per cent nitrogen in amino form in completely hydrolyzed gliadin. Our somewhat lower figure is probably due to a difference in the gliadin preparation used.

† Results at 110° are taken from figures published in the paper on Analysis of Proteins *this Journal* x p 15. The proteins were boiled with 20 per cent hydrochloric acid twenty or more hours until the amino nitrogen became constant.



TABLE IV  
*Egg albumin (Merck)*

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150°	150
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs	1½ hrs	3 hrs
NH <sub>3</sub>	5 5	6 0	7 7	7 5	12 0
NH <sub>2</sub>	69 4	79 5	78 0	78 3	73 4
NH	21 9	12 0	11 6	11 7	13 2
Melanin	3 2	2 6	2 7	2 5	1 4

TABLE V  
*Ox haemoglobin*

TEMPERATURE OF HYDROLYSIS	100	100°	100	110	150	150
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs		1½ hrs	3 hrs
NH <sub>3</sub>	3 7	3 9	4 2	5 2	4 6	5 1
NH <sub>2</sub>	68 9	76 1	75 9	74 0	75 9	74 9
NH	22 0	14 9	15 3	16 6	14 3	15 3
Melanin	5 3	5 2	4 7	3 6	5 3	4 9

TABLE VI  
*Wheat gluten*

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150	150
TIME OF HYDROLYSIS	10 hrs	24 hrs	48 hrs	1½ hrs	3 hrs
NH <sub>3</sub>	17 5	17 8	18 3	18 3	20 8
NH <sub>2</sub>	55 9	62 5	64 9	64 9	63 0
NH	23 5	16 7	14 3	14 1	14 3
Melanin	3 1	2 9	2 5	2 6	1 8

#### SUMMARY

*The percentage of amino nitrogen reaches a definite maximum when acid hydrolysis of a protein is complete, and this maximum is the same whether the hydrolysis occurs at 100° or at 150°* Approximately the same results are obtained by heating at 100° with 20 per cent hydrochloric acid for forty-eight hours as by heating in an autoclave with 3N acid for one and one-half hours as recommended by

Henriques and Gjaldbaek <sup>7</sup> At 100° the amino nitrogen reached its maximum within ten hours in no case, but within twenty-four hours in every case, except that of gluten, which showed a small but definite increase (2.4 per cent) during the second twenty-four-hour period. It appears that with less acid than here used (35 parts of 20 per cent HCl to 1 part of protein) hydrolysis may be somewhat slower, as Osborne and Guest found twenty-four hours' boiling with 10 parts of 20 per cent HCl insufficient to completely hydrolyze casein.

The ammonia, as found by Henriques and Gjaldbaek, does not reach an absolutely definite maximum, but increases the longer hydrolysis is continued. At 150°, prolonging the hydrolysis beyond one and one-half hours caused, in the cases of three of the six proteins, an increase of 2.5–4.5 per cent in the ammonia nitrogen. At 160° the increase of ammonia is still more marked and occurs at the expense of the amino nitrogen. There is much less tendency towards deamination at 100°, the second twenty-four-hour period caused an increase of over 0.8 per cent in the ammonia only in the case of egg albumin (1.7 per cent). Henriques and Gjaldbaek have shown that the well known<sup>8</sup> alteration which cystine undergoes when boiled with hydrochloric acid is accompanied by deamination. That any of the other natural amino-acids are deaminized to an appreciable extent, unless heated under pressure, appears doubtful. That the ammonia, or "amide nitrogen," arises chiefly from the acid amide groups of the asparagine and glutamine in the protein molecule, as indicated by the results of Osborne, Leavenworth and Brautlecht,<sup>9</sup> is consistent with all present results.

<sup>7</sup> *Loc cit*

<sup>8</sup> Mörner *Zeitschr f physiol Chem*, xxxiv, p 207, Van Slyke *this Journal*, x, p 38

<sup>9</sup> *Amer Journ of Physiol*, xxiii, p 194, 1908



# GASOMETRIC DETERMINATION OF FREE AND CONJUGATED AMINO-ACIDS IN THE URINE

By P A LEVENE AND DONALD D VAN SLYKE

(From the Rockefeller Institute for Medical Research, New York )

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The methods for the gasometric determination of total (free + conjugated<sup>1</sup>) and free<sup>2</sup> amino-acid nitrogen in the urine have been outlined in preliminary communications by one of us. The present paper describes the methods in detail, gives some comparative results with the gasometric and formol methods, and presents the results obtained with a number of normal and pathological human urines and with the urines of some dogs under unusual experimental conditions.

For the determination of the total (free and conjugated) amino-acid nitrogen of the urine the gasometric method has decided advantages over the well known Sorensen formol titration.<sup>3</sup> Both methods require heating with acid to hydrolyze the conjugated amino-acids (hippuric acid, peptone, etc.) In the actual determination, however, the gasometric method has an inherent advantage, in that it offers a sharply defined volume of nitrogen gas to be measured at a single burette reading, while the formol titration necessitates the determination of two end points, one with litmus and the other with phenolphthalein, neither point being so sharply defined as those commonly used in volumetric work. The formol titration is further complicated when, as here, the solution to be analyzed is dark in color. This, of course, does not interfere with the gasometric determination. The latter possesses another advantage in accuracy, particularly when small amounts are to be determined, in that the volume of nitrogen gas evolved is five times that of the  $\frac{N}{5}$  barium hydroxide solution required to

<sup>1</sup> Van Slyke *Ber d deutsch chem Gesellsch*, xliii, p 3179

<sup>2</sup> *Ibid*, xliii, p 1685

<sup>3</sup> *Biochem Zeitschr*, vii p 44

titrate the same amount of amino nitrogen. To the formol method also attaches the theoretical objection, pointed out by its originators, that the results will be raised by the presence of weak organic acids, such as oxybutyric, which require more alkali to neutralize them to phenolphthalein than to litmus paper. Whether the error from this source is large enough to affect the significance of results is still undetermined.

For the determination of free (unconjugated) amino-acids, on the other hand, the formol method has a great advantage over the gasometric in that the former is not affected by the presence of urea. Urea, which we have been unable to remove without either hydrolyzing conjugated amino-acids or removing free ones, reacts slowly with nitrous acid, so that a correction has to be introduced for it into the gasometric results. Although this correction can be determined with a fair degree of accuracy, the proportion of urea to amino-acid nitrogen is so great (50 or 100 to 1) that the necessity for the correction more than offsets the advantages which the gasometric method possesses in the absence of urea or the presence of relatively small amounts. A possible error of  $\pm 0.5$  per cent of the total nitrogen of the urine must be allowed for. As the free amino-acid nitrogen normally constitutes only about 1 per cent of the total nitrogen of the human urine, it is evident that the gasometric method is here of value only for determining an abnormally high content of amino-acids. For the detection of a significant increase it is adequate. It also possesses the advantage, over the formol method, that it cannot be affected by the occurrence of abnormally large amounts of organic acids in the urine. For this reason we have described a form of the gasometric method which we have found practicable, although it is not ordinarily so accurate as the formol method for free amino-acid nitrogen, especially in the form in which the latter has been recently developed by Benedict and Murlin.<sup>4</sup>

#### DETERMINATION OF TOTAL (FREE AND CONJUGATED) AMINO-ACID NITROGEN IN THE URINE

In this estimation hippuric acid, peptides, albumin, etc., are hydrolyzed, while urea is decomposed into ammonia and carbon

<sup>4</sup> *Proceedings of the Soc. of Exp. Biol. and Med.*, 1x, May, 1912

dioxide by heating with dilute sulphuric acid under pressure. The ammonia is then boiled off, removing both the preformed ammonia and that from the decomposed urea, and the amino nitrogen is determined in the residual solution. One can obtain very accurate results with this method.

To 75 cc of urine in a large test tube one adds 2.5 cc of concentrated sulphuric acid. The urine is then heated in an autoclave at 175° for an hour and a half.<sup>5</sup> It is then transferred to a 300 cc Jena Erlenmeyer, 5 to 6 grams of powdered calcium hydrate are added, with a piece of paraffin to prevent foaming, and the mixture is boiled until the vapors no longer turn red litmus blue. If necessary, water is added to keep the solution from going dry before this point is reached. Usually all the ammonia is driven off within about an hour, but sometimes a urine is encountered which requires twice as long.

The hydrolyzed urine is filtered through a folded filter into an evaporating dish, the precipitate, consisting of calcium sulphate, hydrate, etc., being washed ten times with hot water. The filtrate is concentrated on the steam bath to a volume of a few cc and filtered from the small amount of calcium salt, which has settled out, into a 25 cc measuring flask. One can then perform duplicate amino determinations in the usual way<sup>6</sup> on 10 cc portions. The total amino nitrogen in normal urines appears to be quite constant at 1.5 to 2.5 per cent of the total nitrogen. More than 3 per cent we have encountered only in pathological cases, in which the high results are usually due to albumin.

About a full working day is required by the above method to prepare the urines for the final determination, but during a large part of the time (heating in autoclave, concentrating on water bath) but little attention is required. As many urines can be prepared at the same time as the autoclave will hold tubes, the ammonia from all being boiled off at the same time on a hot plate.

The following results indicate the accuracy of the method. Two

<sup>5</sup> The quantitative decomposition of urea under these conditions was demonstrated by Benedict and Gephart (*Journ Amer Chem Soc*, 1909) and confirmed by Levene and Meyer (*ibid*). The new method of Benedict (decomposition of the urea in a  $\text{KHSO}_4$  melt) is not applicable here.

<sup>6</sup> Van Slyke this *Journal*, 17, p 185, cf., also, preceding article on improvements in the method.

75 cc portions of normal urine were analyzed as described, 0.1835 gram of Kahlbaum's alanine having been added to one portion, the other serving as control. The solutions, at the end of the analysis, were brought to 25 cc and the amino nitrogen determined, as usual, in 10 cc portions.

	N	TEMPERATURE	PRESSURE	NH <sub>2</sub> -N	ALANINE N FOUND	ALANINE N PRESENT
	cc	degrees C	mm	mgm	mgm	mgm
Urine I	9.0	21	758	5.09		
Urine II	9.0	21	758	5.09		
Urine—Alanine I	29.4	20	757	16.67	11.58	11.55
Urine—Alanine II	29.1	19	756	16.56	11.47	11.55

The fact that the autoclave treatment effects a practically complete cleavage of hippuric acid is shown by the following. One gram of sodium hippurate was dissolved in 75 cc of water with 2.5 cc of concentrated sulphuric acid and heated one and a half hours in the autoclave. The solution was then diluted to 100 cc and determinations of the total nitrogen present made upon 20 cc portions, those of the amino nitrogen upon 10 cc portions.

TOTAL NITROGEN		AMINO NITROGEN	
Found	Calculated	Found	Calculated
0.0693	0.0696	0.0685	0.0696

The complete hydrolysis of a soluble protein effected by the autoclave treatment is shown by the following results. One gram of air-dried proto-albumose was dissolved in 75 cc of water, the remainder of the treatment being that described above in the hippuric acid experiment. The total nitrogen found was 0.1484 gram, the amino nitrogen was 0.1025 gram, or 69.1 per cent of the total. In proto-albumose, completely hydrolyzed with boiling hydrochloric acid, 69.13 per cent of nitrogen in amino form was found.<sup>7</sup> While all proteins may not be hydrolyzed with the same degree of absolute completeness, it may be stated with confidence that the amount of unhydrolyzed albumin left in a urine after the autoclave treatment for destruction of urea will be minimal.

<sup>7</sup> Levene, Van Slyke and Birchard *this Journal*, x, p. 68, 1911.

## DETERMINATION OF FREE AMINO-ACID NITROGEN

The amino-acids, when treated with nitrous acid under the conditions used for amino nitrogen determination,<sup>8</sup> give off in two to five minutes 100 per cent of their nitrogen. Urea under the same conditions requires about eight hours to react completely. In two to five minutes it gives off only 3-4 per cent of its nitrogen, and the reaction continues for a considerable time without appreciable change in the volume of nitrogen evolved per minute. This rate is also unaffected by the presence of reacting amino-acids. By taking advantage of the slow and regular rate at which urea reacts, one can determine in its presence the nitrogen of the almost instantaneously reacting amino-acids, making a correction for the small percentage of urea which reacts within the short time required for the determination.

The ammonia must first be removed, and the determinations of  $\text{NH}_3$ - and  $\text{NH}_2$ -nitrogen can be conveniently combined. We found distillation with calcium hydrate under diminished pressure the most satisfactory means for removing and determining the ammonia. The apparatus and technique used are the same as described for the determination of amid nitrogen in proteins.<sup>9</sup> Fifty or 100 cc of urine, with an equal volume of 95 per cent alcohol and enough of a 10 per cent calcium hydrate suspension to give a strongly alkaline reaction, are placed in a double-necked distilling flask of 500 cc or 1 liter capacity, and distilled as described, until all the alcohol has been driven off. This point is reached in twenty to thirty minutes and is indicated by vigorous foaming of the solution. It is essential that the removal of the ethyl alcohol be practically complete, as when mixed with nitrous acid it gives off vapors or gases which are only with difficulty absorbed by the permanganate solution.

The distillation being finished, the calcium hydrate and salts are dissolved by the addition of 2-3 cc of glacial acetic acid. The urine is now transferred to a measuring flask and made up to its original volume of 50 or 100 cc. Of this, 10 cc portions are used for the amino determination. It is of no advantage, except in

<sup>8</sup> Van Slyke Quantitative Determination of Aliphatic Amino Groups, this *Journal*, ix, p 185, xii, p 275

<sup>9</sup> Van Slyke this *Journal*, x, p 21



unusually dilute urines, to concentrate the urine so that relatively more can be used for the determination. The limit of accuracy of the determination depends upon that of the urea correction, and the absolute error of this is increased in proportion to the concentration of the urea, so that there is little or nothing to gain in percentage accuracy by increasing the volume of gas to be measured.

The amino determination and the correction for the urea are now made as follows. One amino determination is performed, the duration of the reaction being the shortest time in which, at the prevalent temperature, amino-acids will react completely. Immediately thereafter the determination is repeated under the same precise conditions, except that the reaction is continued for exactly twice as long. The *increase* in volume obtained, by doubling the reaction time, represents the amount of nitrogen evolved from the urea during half the time of the second determination, or, the entire duration of the first. It is, therefore, the correction which must be subtracted from the first result in order to leave only the nitrogen obtained from amino-acids. Amino-purines and aminopyrimidines react slowly, like urea, and consequently if present will not be determined with the amino-acids.

In the preliminary description of the method, when the original hand-manipulated apparatus was employed, six and twelve minutes were the durations used for the two determinations. The more efficient action of a rapidly shaken motor-driven apparatus enables one to cut the time down to the following: for temperatures of 15 to 20°, four and eight minutes, for 20 to 25°, three and six minutes, for over 25°, two and four minutes. During the shorter of the two determinations the reacting solution is shaken constantly and rapidly. During the longer determination it may either be shaken constantly, or, during the first half minute (to mix the solutions) and the last two minutes (to drive out all the nitrogen formed). It is particularly essential that the shaking should be rapid during the last minute, in each case, so that the nitrogen formed up to the end of the interval may be at once driven out.

The volumes of acetic acid and nitrite solution used and the volume of nitrous acid solution in the deaminizing vessel when the urine is run in, must here be accurately the same in each pair of determinations, and not roughly measured, as may be the case in ordinary amino analyses. Exact measurement of the reaction

time is also essential and is easy to attain with a stop watch. In order to make the starting points for the time measurement as sharp as possible, we run the urine into the deaminizing vessel rapidly until only a few tenths of a cubic centimeter remain to be added. The watch is then started, while the remaining small portion of the solution is added more cautiously.

The approximate constancy of the reaction rate of urea during at least the first eight minutes, at a given temperature, is illustrated by the following figures. The solution used contained 2 per cent of urea, about as much as an average human urine

DURATION OF REACTION	TEMPERATURE	N	N PER MINUTE
minutes	degrees C	cc	cc
3	25	9.8	3.27
6	25	19.5	3.25
4	25	13.2	3.30
8	25	26.2	3.28
3	19	5.8	1.93
3	19	6.0	2.00
6	19	12.0	2.00
6	19	11.6	1.93

The following determinations indicate that approximate results for amino-acid nitrogen can be obtained in the presence of a great excess of urea, making the correction for urea in the manner de-

DURATION OF REACTION	TEMPERATURE	PRESSURE	N	CORRECTION FOR UREA N	N FROM ALANINE	CALCULATED N FROM ALANINE
minutes	degrees C	mm	cc		cc	cc
3	24	752	22.6	8.7	13.9	14.2
6	24	752	31.3			
3	24	752	23.1	8.5	14.6	14.2
6	24	752	31.6			
3	24	752	23.3	8.4	14.9	14.2
6	24	752	31.7			
2	29	760	23.8	9.7	14.1	14.3
4	29	760	33.5			
2	29	760	23.6	9.7	13.6	14.3
4	29	760	33.3			
2	29	760	23.8	9.6	14.2	14.3
4	29	760	33.4			

scribed above. The solution used contained 2 grams of urea and 0.5 gram of alanine per 100 cc. The proportion of urea nitrogen to amino-acid nitrogen was 12.1. Three pairs of determinations were performed on each of two different days, on which there were 5° difference in the room temperatures. The solution was kept at 0° during the interval in order to prevent the rapid deamination which bacteria can accomplish.

As a further control, 200 cc of normal human urine, containing 0.574 gram of nitrogen per 100 cc, were freed from ammonia as previously described (ammonia nitrogen = 0.021 gram per 100 cc). Determination of the free amino nitrogen in this urine showed that the amount present was too small to detect. Two-minute determinations at 29° gave 4.0, 4.0, 4.3 cc of N, four-minute determinations gave 8.1, 8.4, 8.4 cc. One-half gram of alanine was dissolved in 100 cc of the urine, which was then freed from ammonia, brought back to 100 cc, and used for the following determinations. The temperature was 19°, pressure, 760 mm.

DURATION OF REACTION	N	N FROM AMINO-ACIDS	CALCULATED FOR ALANINE PRESENT	ERROR
minutes	cc	cc	cc	cc
3	17.8	13.5	13.8	-0.3
6	22.1			
3	18.0	13.3	13.8	-0.5
6	22.7			
3	18.3	14.1	13.8	+0.3
6	22.5			

#### ANALYSES OF HUMAN NORMAL AND PATHOLOGICAL URINES

The analyses tabulated were carried out as described above except that most of the determinations of free amino-acid nitrogen were performed by the earlier modification<sup>10</sup> used before the improved apparatus for determining amino nitrogen<sup>11</sup> was available. When the results for total amino-acid nitrogen were checked with Sorensen's formol method, 10 cc of the 25 cc of final solution were used for the gasometric determination and an equal portion for

<sup>10</sup> *Ber d deutsch chem Gesellsch*, xiv, p 1685, 1911

<sup>11</sup> *This Journal*, xii, p 275

the formol titration. The formol results, though in some cases not appreciably different from the gasometric, were as a rule markedly higher, the maximum difference being equal to 1 per cent of the total nitrogen of the urine.

The higher results for the formol method may be due to the presence of organic acids, which are prejudicial to its accuracy. It appears doubtful that, as a rule, this source of error is sufficient to cause a clinically or scientifically significant deviation in the formol results, although a positive statement can not be made until the formol and gasometric methods have been used together on a larger and more varied collection of urines.

The total (free + conjugated) amino-acid nitrogen bears a fairly constant relation to the total nitrogen. All the normal and most of the pathological urines contain not less than 1.1 nor more than 2.8 per cent of their nitrogen in this form. The only cases in which 3 per cent was exceeded were those of nephritis and the arthritic cases, which also usually have albumen in the urine. The probability that all the abnormal amino nitrogen obtained was freed by cleavage in autoclave is shown by the later cases, in which the free amino nitrogen was also determined. This is not high even when the total amino nitrogen of a nephritic reaches 2.4 per cent. Aside from nephritic cases, one cancer case gave a very high figure, 6.07 per cent. Unfortunately we had no determination of the free amino nitrogen in this case. Two cases of liver cirrhosis gave entirely normal values.

The free amino nitrogen never exceeded 1.5 per cent in the limited number of cases in which it was determined.

NO	CASE	TOTAL N PER 100 CC URINE	TOTAL NH <sub>2</sub> PER 100 GRAMS N (Gasometric)	TOTAL NH <sub>2</sub> PER 100 GRAMS N (Formol)	FREE NH <sub>2</sub> PER 100 GRAMS N (Gasometric)
1	Normal	0.300	2.50		
2	Normal	0.808	1.95	2.45	
3	Normal	0.925	1.80	2.76	
4	Normal	0.780	1.86	2.45	
5	Normal	1.336	2.02	2.42	
31	Normal	0.941	1.77		0.3
35	Normal	1.043	1.14		

NO	CASE	TOTAL N PER 100 CC URINE	TOTAL NH <sub>2</sub> PER 100 GRAMS N (Gasometric)	TOTAL NH <sub>2</sub> PER 100 GRAMS N (Formol)	FREE NH <sub>2</sub> PER 100 GRAMS N (Gasometric)
36	Normal	0 623	2 81		1 5
37	Normal	0 491	2 12		0 3
38	Normal	1 470	1 28		0 5
39	Normal	0 613	2 70		0 5
40	Normal	0 624	2 41		0 2
50	Normal	1 067	1 28		
5	Cancer of antrum	0 347	2 10		
32	Carcinoma of breast	0 702	6 07		
44	Carcinoma of larynx	1 485	2 29		1 5
51	Carcinoma of larynx	1 382	2 38		
2	Arthritis	0 585	2 39		
6	Arthritis	0 580	5 56		
11	Arthritis	0 363	3 25		
12	Arthritis	1 201	3 60	3 97	
13	Arthritis	1 112	2 26	2 34	
14	Arthritis	0 901	2 17	2 73	
15	Arthritis	0 454	1 90		
20	Gout	0 568	3 87	3 89	
1	Multiple sclerosis	0 655	1 65		
7	Multiple sclerosis	0 396	2 51		
8	Multiple sclerosis	0 943	1 32		
3	Paralysis agitans	1 813	1 54		
16	Anterior poliomyelitis	1 283	1 34	1 83	
46	Syphilis	0 745	2 92		
55	Hypophysis disease	0 364	1 55		
4	Progressive musc dystrophy	0 728	1 92		
9	Progressive musc dystrophy	1 654	1 82		
17	Progressive musc dystrophy	0 959	2 33	2 48	
33	Progressive musc dystrophy	1 470	1 83		
41	Progressive musc dystrophy	1 267	2 03		1 1
47	Progressive musc dystrophy	0 415	1 82		1 5
49	Progressive musc dystrophy	1 923	1 37		0 5
52	Progressive musc dystrophy	1 210	1 61		0 3
54	Progressive musc dystrophy	0 434	1 21		0 8
34	Liver cirrhosis	0 889	1 92		0 6
45	Liver cirrhosis	0 455	1 68		0 8
42	Nephritis	0 781	2 00		1 2
43	Nephritis	0 654	24 30		1 2
53	Nephritis	1 497	7 53		2 0
56	Nephritis	0 364	1 55		
57	Nephritis	1 120	1 62		

*Analyses of dog urines*

	TOTAL N PER 100 CC URINE	TOTAL NH <sub>2</sub> PER 100 GRAMS N (GASO- metric)	TOTAL NH <sub>2</sub> PER 100 GRAMS N (Formol)	FREE NH <sub>2</sub> PER 100 GRAMS N (GASO- metric)
Normal dog (urine diluted)	0 625	1 67	1 79	
Normal dog (urine diluted)	1 294	1 05	0 92	
Dog fed glycine anhydride (urine diluted)	0 168	39 80	39 20	
*Dog poisoned 10 days with chloro- form	2 711	1 78		
*Dog poisoned 11 days with chloro- form	1 523	1 92		
*Dog poisoned with chloroform and phosphorus (urine diluted)	0 893	1 59		
Normal dog fed 2 grams <i>dl</i> -phenyl- alanine (urine diluted)	0 226	8 50		
Normal dog fed <i>dl</i> -aspartic acid (urine diluted)	0 125	2 38		
Gastrectomized dog, normal diet (urine diluted)	0 188	1 91		
Gastrectomized dog, normal diet (urine diluted)	0 406	1 90		
Gastrectomized dog, normal diet (urine diluted)	0 219	2 78		
Gastrectomized dog, normal diet (urine diluted)	0 415	2 02		
Dog which had received 12 grams of <i>dl</i> -alanine intravenously 20 min- utes before catheterizing	0 445	53 00		52 40

\*Urine furnished by Dr Dochez

It was a cause for surprise that the dogs poisoned with chloroform and with chloroform and phosphorus, gave urines containing only the normal small percentage of amino nitrogen, although the livers of these animals were degenerated to an extreme degree. These results indicate that even rapid degeneration of the liver may not result in excretion of amino-acids. The two cases of human liver cirrhosis also showed only normal amino nitrogen in the urine.

The non-utilizability of glycine-anhydride in the organism is strikingly shown by the tremendous excretion of conjugated amino-

acid nitrogen following its administration The permeability of the kidney for amino-acids, when injected directly into the blood, is shown by the last result It does not indicate that the organism fails to utilize amino-acids even thus injected, however, for only 1.5 grams of the 12 grams of alanine injected were excreted unchanged

# ON THE OCCURRENCE OF METHYL GUANIDINE IN THE URINE OF PARATHYROIDECTOMIZED ANIMALS

By W F KOCH

(From the Laboratory of Physiological Chemistry, University of Michigan)

(Received for publication, July 2, 1912)

The experiment described in this paper was designed to determine whether guanidine could be detected in the urine of animals subjected to thyro-parathyroidectomy

On May 8, the thyroid and parathyroid glands were removed from a dog weighing 18.5 kgms. On May 10, at 11 a.m., fibrillary twitchings were noted in his body muscles. The symptoms increased in violence until at 9.15 p.m. he died in severe tetany. During this period he excreted 2250 cc. of urine, which was examined as follows:

The kynurenic acid was removed with hydrochloric acid. The protein-like substances were precipitated from the acid filtrate with 20 per cent tannin solution. The tannin excess was removed with barium hydroxide, the barium excess with sulphuric acid and the excess of the latter with lead oxide. The resulting filtrate was then treated with a hot saturated solution of sodium acetate and mercuric chloride until no more precipitate formed, according to the method of Engeland<sup>1</sup>. The precipitate was then filtered off, washed with a cold saturated solution of mercuric chloride and sodium acetate, treated with hot dilute hydrochloric acid and the soluble portion filtered from the insoluble. The filtrate was then decomposed with hydrogen sulphide and filtered. The resulting filtrate was evaporated to a thick syrup and extracted with methyl alcohol. The extract was then filtered from inorganic salts. The methyl alcohol was evaporated from the filtrate, the residue extracted with ethyl alcohol, filtered from insoluble residue and

<sup>1</sup> *Zeitschr f physiol Chem*, lvn, p. 49



again extracted with alcohol. This process was repeated until no more creatinine and ammonium salts were dissolved. The resulting alcoholic solution was treated with 20 per cent platonic chloride until no more precipitate occurred. The precipitate was then filtered off.

The filtrate was decomposed with hydrogen sulphide, the platinum sulphide removed, the filtrate evaporated to a thick syrup and treated with a 30 per cent solution of gold chloride.

The platonic precipitate was suspended in water, decomposed with hydrogen sulphide and the platinum sulphide filtered off. To the resulting liquid a 30 per cent solution of gold chloride was added.

From the gold solution from the platonic filtrate, after standing in the desiccator for several days, a rich crop of beautiful needles formed and were filtered off. In the course of another day a second precipitate of needles and cubes occurred. These were separated mechanically as far as possible and fractionally crystallized. From the mother liquid, on further standing, another precipitate, apparently only of cubes and plates, formed. Both fractions of needles on recrystallization showed the same melting point, namely,  $198^{\circ}\text{C}$  (uncorrected). They possessed also similar solubilities in water and ether and were therefore combined.

From the gold solution of the platinum precipitate a crop of needles crystallized. These were filtered off. In the course of twenty-four hours another precipitate of both needles and rhomboid plates was obtained. The needles and plates were fractionally crystallized and both yields of needles, which possessed the same melting point ( $198^{\circ}\text{C}$ ), were united.

Owing to the similarity in the melting points and the solubilities of the needles of both the platonic filtrate and precipitate, they were united and weighed, the total yield being 4.3 grams. They were recrystallized from hot dilute hydrochloric acid and a portion dried in the air and later at  $90^{\circ}$  for melting point and gold content determinations. At  $198^{\circ}$  they melted to a red brown liquid.

ANALYSIS (a) 0.084 gram substance gave 0.0402 gram Au  
(b) 0.1635 gram substance gave 0.078 gram Au

	Calculated for		Found
	$\text{C}_2\text{H}_7\text{N}_3\text{HClAuCl}$	(a)	(b)
Au	47.7	47.86	47.71

Complete analyses have not been made but the melting point and gold percentage identify the substance as methyl guanidine aurochloride

From the remaining gold solution of the platonic precipitate after standing several days in the desiccator and after cooling at a temperature of 30° for twelve hours a yield of about 2 grams of crystals was obtained. These were found to melt in a peculiar manner after recrystallization and drying. At about 180° they gradually became cloudy and between 207° and 208.5° melted to a brown oil. Finally about a gram of prisms melting at 310°C were obtained. They will be further studied.

Methyl guanidine was first isolated from normal human urine by Kutscher and Lohman<sup>2</sup>. From 100 liters they obtained about 4 grams as the aurochloride. Later Engeland<sup>3</sup> isolated 2.1 grams of the gold salt from 28 liters of normal urine. From 11 liters of normal dogs' urine Achelis<sup>4</sup> isolated 0.122 gram as the picrolonate, corresponding to about 0.04 gram of the free base per liter of urine. These results correspond to about 0.07 gram of the gold salt per liter of normal urines, both of man and dog. In the case noted above the yield was considerably in excess, namely, 1.9 grams as the gold salt per liter. The greater part was probably excreted on the day of the animal's death since no symptoms were noticeable until ten hours before death. During the last five hours he passed only a few cubic centimeters of urine. On microscopic examination of the kidney, the cortex was found to be very hyperemic and hemorrhagic. Many of the glomeruli had become blood islands, with complete loss of Bowman's capsule. It is not surprising that under these conditions little urine was passed during the hours just preceding death, a probable indication that, since the kidney function had been inhibited, whatever methyl guanidine might have been formed must have accumulated in the blood.

Further investigation is being carried on concerning the extent to which methyl guanidine is responsible for the symptoms and death of the parathyroidectomized animals and concerning the nature of the other bases which our work has shown to be present.

I take pleasure in expressing my gratitude to Dr. Vaughan, Dr. Huber and Dr. Novy for their kind interest and many helpful favors rendered during the course of the work.

<sup>2</sup> *Zeitschr f physiol Chem*, xlix, p 81

<sup>3</sup> *Ibid*, lvii, p 49

<sup>4</sup> *Ibid*, l, p 10



# ANIMAL CALORIMETRY

## FIRST PAPER

### A SMALL RESPIRATION CALORIMETER

#### PLATES II-IV

By H. B. WILLIAMS

(From the Physiological Laboratory, Cornell University Medical College, New York )

(Received for publication, July 17, 1912 )

It is the purpose of this paper to describe briefly an apparatus for studying the energy metabolism and gaseous exchanges in infants and small animals, and to give an account of the control experiments which have been made with the apparatus in order to determine the magnitude of the errors to which the various measurements are liable

The apparatus is a constant-temperature, water-cooled calorimeter

The removal of the heat is brought about by a constantly flowing stream of water. Considered as a respiration apparatus, it is of the closed circuit type of Regnault and Reiset<sup>1</sup>. In its general plan it is based on the type of apparatus developed by Atwater and Rosa<sup>2</sup> and embodies many of the structural features which have been introduced by Benedict<sup>3</sup>. A complete and detailed description of the apparatus would expand the paper beyond the scope of a journal article. The plans of the larger calorimeters at Middletown and at Boston have been described in great detail in

<sup>1</sup> Regnault and Reiset *Ann d Chem u Pharm*, lxxiii, pp 92, 129, 257, 1850

<sup>2</sup> Atwater and Rosa *Description of a New Respiration Calorimeter*, U S Department of Agriculture Bull 63, 1899

<sup>3</sup> Atwater and Benedict *A Respiration Calorimeter, with Appliances for Direct Determination of Oxygen*, Carnegie Institution of Washington Publication 42, 1905

various bulletins of the Department of Agriculture and of the Carnegie Institution of Washington <sup>4</sup> It seems, therefore, that a description of the apparatus in general terms, with the assistance of photographs, will make clear our experimental procedure to anyone who is at all familiar with the publications above referred to From these publications also may be obtained a full account of the methods of calculating the results Certain special features of the small calorimeter will be described in greater detail

As in the larger instruments of Atwater, Rosa and Benedict, the respiration chamber consists of a rectangular copper box The inside dimensions are approximately 66 cm wide, 76 cm high and 96 cm long The capacity of the chamber is approximately 480 liters

In the front of the chamber is a window for the entrance of the subject This window is closed with two glass plates and hermetically sealed with wax during the experiments The copper chamber is supported within a framework of structural iron, which gives it great rigidity

The actual metallic connection between the copper box and the iron frame has, however, been limited to a relatively small number of points so as to diminish the tendency for heat to pass to and from between them in case of a difference in temperature Surrounding the iron framework is a second wall of zinc Seventy-two wooden spools, each carrying four thermo-couples, pass between the copper and zinc walls and are used after the method of Rosa to indicate differences in temperature between the walls of the inner and outer chambers The passage of heat through these walls in either direction is avoided by altering from time to time the temperature of the zinc, and with it that of the iron work with which it is directly connected, so that no sensible difference in temperature shall prevail between them and the copper wall of the chamber Under these circumstances the gradient is nearly zero and there will be but little tendency for heat-energy to pass in either direction

For that reason the wall is said to be adiabatic The thermo-couple system serves to indicate to the operator when to change

<sup>4</sup> Atwater and Rosa *loc cit*, Atwater and Benedict *loc cit*, Benedict and Carpenter *Respiration Calorimeters for Studying the Respiratory Exchange and Energy Transformation of Man*, Carnegie Institute of Washington Publication 123, 1910

the temperature of the zinc wall and how nearly alike the zinc and copper are at any moment To permit this control to be exercised the parts just described are enclosed within a third wall composed of a sheathing of asbestos and cement, lined with a layer of cork several centimeters thick

Between the inner surface of the cork and the zinc wall an air space of about 6 centimeters is left Within this air space runs a system of copper tubes through which cold water is allowed to circulate during the entire experimental time This water is so cold that it can maintain the zinc wall and the iron frame always cooler than the lowest temperature which it is proposed to allow the copper chamber to assume Parallel to the copper tubing runs a system of manganin resistance wires, insulated with enamel and supported on porcelain knobs By passing an electrical current through these wires the effect of the cold water in the tubes can be wholly or partially neutralized The extent of the heating effect can be accurately controlled by the operator by the manipulation of a ballast resistance in series with the wire As in the later apparatus of Benedict, the air space between the outer and middle walls has been divided up into compartments, each with its own separate system of water pipes and heating wires The thermo-couple system can be made to indicate the condition of the walls of the top, sides and bottom, separately, or of the entire wall

In this instrument the adiabatic control has proven adequate, but the presence of so large an amount of structural iron between the walls, while giving great rigidity, makes necessary great care in manipulation to avoid errors, and in the construction of such apparatus it would probably be better to use less iron, or even preferably none at all, and to construct the framework of materials which have less heat capacity and less conductivity The extent to which the adiabatic control is able to prevent gain or loss of heat in spite of the presence of so much structural iron in the frame will be made evident in the discussion of the control experiments

To maintain the temperature of the inner chamber at a constant level with a source of heat inside it, the heat must be removed as fast as it is eliminated by the source and no faster This is accomplished by permitting water to flow at a constant rate through a system of piping within the copper chamber The temperature

of this water is so regulated as to secure the removal of heat at the rate at which it is given off by the subject of the experiment. The total amount of water which flows during an experimental period is collected and weighed. The temperature of the water is measured at the point where it enters the inner chamber and at the point where it leaves by means of two specially calibrated mercurial thermometers which are read every four minutes.<sup>5</sup> These thermometers can be read to  $0.01^{\circ}\text{C}$ . The average difference in temperature multiplied by the weight in kilograms of the water used during an experimental period is a measure of the heat removed *in this manner*<sup>6</sup> in large calories at the mean temperature of the flowing water.

As the standard calorie for work of this nature, Atwater and Rosa adopted the amount of heat required to warm one kilogram of water from  $19.5^{\circ}\text{C}$  to  $20.5^{\circ}\text{C}$ . This amount of heat is different from that required to warm a kilogram  $1^{\circ}\text{C}$  at  $0^{\circ}$  or at  $15^{\circ}$  on account of the fact that the specific heat of water varies with change of temperature.

In the neighborhood of  $20^{\circ}$  this variation is slower than at temperatures somewhat higher or lower. In case the mean temperature of the flowing water differs much from  $20^{\circ}$  it would be necessary in accurate work to make a correction for the variation of the calorie, but in all of our experiments as well as in the controls the mean temperature has been very little different from  $20^{\circ}$  and such a correction is superfluous.

Despite the effort to maintain a perfect balance between the rate of heat elimination and the rate of its removal from the calorimeter, there will in general be slight changes in temperature of the copper wall. To measure these changes a system of electrical resistance thermometers enclosed in small copper boxes has been soldered to the outside of the copper chamber between it and the zinc wall. These are six in number and being widely distributed over the surface they integrate the temperature of the

<sup>5</sup> The water thermometers were calibrated by comparison with standard Richter thermometers. The standards can be read with a glass to  $0.001^{\circ}$  and were certified by the Physikalische technische Reichsanstalt to be "ohne wesentliche Fehler" in 1910.

<sup>6</sup> Part of the heat may be removed in the ventilating air current as latent heat of vaporization of water. See below.

wall Since, in raising or lowering the temperature of the zinc wall to maintain it at the same temperature as that of the copper wall, the zinc and iron frame work may receive (or give up) heat from either the copper wall or the outer air space, the hydrothermal equivalent of the apparatus cannot be determined by calculation, but must be found experimentally The method of determining this factor has been described by Atwater and Benedict<sup>7</sup> and by Benedict and Carpenter<sup>8</sup>

The determination of the amounts of carbon dioxide and water vapor eliminated by the subject and the quantity of oxygen absorbed is accomplished in much the same manner as in the larger instruments by maintaining with a rotary pump a continuous flow of air into and out of the apparatus As the air is removed it passes successively through a sulphuric acid wash bottle to remove the moisture, then over moist granular soda-lime to remove carbon dioxide and finally through another sulphuric acid wash bottle to remove moisture which the air current may have taken up from the moist soda-lime

The air thus purified is returned to the chamber It is evident that on account of absorption of oxygen by a subject (animal or alcohol lamp), the volume of gas within the apparatus, provided no change in temperature or pressure occurred, would become continually less and that it would contain a constantly diminishing percentage of oxygen Following the method employed in the calorimeters at Boston we have provided that the pressure within the apparatus shall always be the same as that of the external atmosphere, by connecting the interior of the box with a large tambour, covered with a soft rubber cap To this is attached a pointer which serves as a volume indicator as well as for the purpose of automatically admitting oxygen from a cylinder by closing an electrical contact whenever the volume of the rubber bag diminishes below a predetermined point By this means the apparent volume of the apparatus is maintained nearly constant throughout the experiments The current of air as it leaves the apparatus is made to pass over one end of a system of thermo-couples and the entering air over the other end The entering air is then cooled or heated as may be necessary to keep its temperature always the

<sup>7</sup> *Loc cit*, p 152

<sup>8</sup> *Loc cit*, p 52



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<sup>7</sup> *Loc cit*, p 152

<sup>8</sup> *Loc cit*, p 52

same as that of the leaving air and so prevent gain or loss of heat. Within the chamber is a system of electrical resistance thermometers for determining the average temperature of the air.

From the above general description it can be seen that the apparatus is very similar in principle and construction to the larger instruments of its type, but in the carrying out of the details a number of modifications were found necessary or desirable. We may consider first the changes which have been made in the part of the apparatus which has to do with the gas analysis.

It was appreciated at the outset that the large porcelain absorbers used by Benedict for removal of water from the air current were so heavy that it would be quite impossible to weigh them with sufficient accuracy to determine the small quantities of water it was proposed to measure. After considering various expedients, the form of glass wash bottle shown in figure 1 was designed. The air current enters through a central tube which opens below within a rosette-like expansion. The lower edge of this tube is about 3 mm from the bottom of the rosette. The bottom of the rosette is about 4 or 5 mm from the bottom of the bottle and is pierced near the edge by a series of holes *B* arranged in a circular row. On the upper aspect of the rosette is a second series of holes arranged in a circular row marked *A* in the figure.

The air current on striking the bottom of the rosette is partly broken up and deflected upward through the acid within the rosette. It escapes through the series of holes *A* which have the effect of breaking up the larger bubbles into much finer ones. No air escapes through the holes *B* in the bottom of the rosette, but as the air current continually forces acid with it through the holes *A* a stream of acid constantly enters the rosette through the holes *B*. The air leaves the bottle through a tube at the top with a bulbous expansion containing an inner bulb marked *C*. This inner bulb is pierced along its horizontal diameter by a ring of holes. The bulb *C* acts as a baffle to prevent spattering of the acid into the tube leading from the bottle.

The series of holes *A* and the holes in *C* are of such a diameter that their total area is equal to or slightly in excess of the area of the section of the large inflow and outflow tubes. The latter have an internal diameter of about 15 mm.

The height of the bottles inside is about 150 mm and the diam-

eter 95 mm When filled for use they contain 750 cc of sulphuric acid and weigh about 1500 grams They can be weighed to 0.01 gram on the balance ordinarily used and, if desired, by using one of the large balances of Sartorius the weighing error could be reduced still more The mixture between the air and acid in these wash bottles is extremely intimate and their ability to remove from the air current large quantities of water rapidly and thoroughly has more than fulfilled our expectations With the air current flowing at the rate of 30 liters per minute a single bottle filled

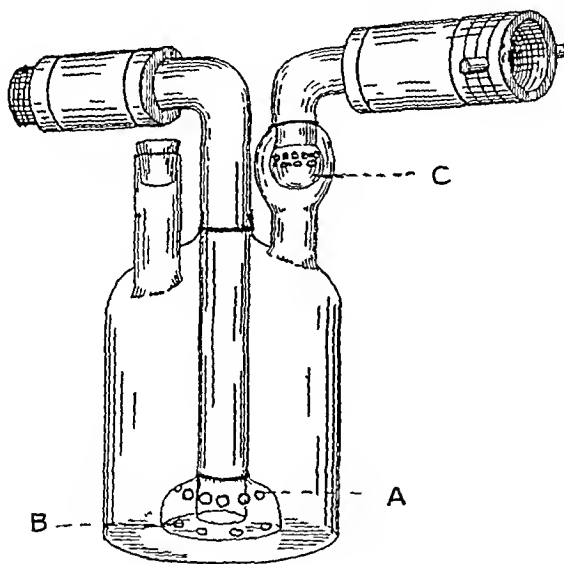


FIG 1 GAS WASH BOTTLE

with fresh acid has absorbed as much as 25 grams of water per hour without permitting a weighable amount to pass it, as was shown by the failure of a second bottle in series with it to gain in weight Under ordinary working conditions the amount of water seldom exceeds 10 grams per hour and the acid is systematically renewed before it has become sufficiently spent to endanger loss of water In addition to the efficiency tests made in this laboratory, Dr Francis G Benedict of the Nutrition Laboratory at Boston has kindly examined and tested one of these bottles and has been able

to confirm their efficiency in absorbing rapidly the amounts of water for which they were designed. These bottles are now extensively used in the small Benedict respiration apparatus.<sup>9</sup>

The size of the soda-lime cans was also diminished in order to secure greater accuracy in weighing,<sup>10</sup> and for the oxygen a small steel cylinder, weighing about 5.5 kgms. has been used.

The oxygen and soda-lime can be weighed to 0.01 gram. The small oxygen cylinder filled at a pressure of 75 atmospheres will suffice for six to eight hours of experimenting on a dog weighing 16 kilos.

As in the larger calorimeters the admission of the oxygen has been made automatic by permitting the rubber tension equalizer to operate an electrical contact which would actuate an electromagnet. The admission has been made more sensitive by attaching the contact to the long pointer which magnifies the movements of the rubber bag and as a result very little fluctuation in the apparent volume of the system occurs. Oxygen is admitted directly into the entering air pipe, an arrangement which insures immediate diffusion within the chamber. Only at the end of the periods when the apparent volume of the system is adjusted to the standard point, is oxygen admitted to the rubber bag. At such times the bag is momentarily shut off from the rest of the system and the quantity of oxygen admitted is never great. Instead of using a Toepler manometer and adjusting the bag to constant tension, a scale at the end of a long pointer attached to the bag has been used to indicate the volume. To determine whether the indications of this pointer were reliable the following experiment was made.

A two-necked bottle was connected on one side with the rubber bag and on the other with a burette containing mercury.

By allowing mercury to flow from the burette into the bottle a volume of air equal to that of the mercury will be forced into the bag. The volume of mercury used can be read off on the burette.

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<sup>9</sup> Benedict and Homans *Journ. of Med. Research*, xxii (new series), p. 423. These bottles were blown for us by E. Machlett and Sons, East 23rd Street, New York.

<sup>10</sup> Since this paper was begun the use of metal cans for soda-lime has been discontinued and at the suggestion of Dr. Benedict large glass bottles are employed instead.

It was found that the amount of mercury necessary to displace the volume of air required to move the pointer from any particular point on the scale to any other specified point was always the same within 0.5 cc. Over a wide range the deflections of the pointer were strictly proportional to the change in volume.

The weight of the pointer is counterbalanced so that it produces no compression of the bag. The error in adjusting to constant volume by this method is very much within the error of weighing the amount of oxygen used. The pumping action of the rubber bag during the periods of one hour between adjustments is quite sufficient to insure thorough diffusion of the oxygen thus admitted to the bag throughout the system.

In the apparatus for measuring heat several changes have been made. The air and wall thermometers have been wound with pure nickel wire which has a higher rate of resistance change with temperature than copper in the proportion of 3 to 2. The air thermometers have been given a flat form to economize space.

These thermometers were made by the Leeds and Northrup Company of Philadelphia. They were calibrated before installation in an oil bath in terms of the gas thermometer scale and have been given the same resistance and the same coefficient of resistance change per degree so that identical bridge readings on the two sets would mean that the temperature of air and wall were the same. This has been found helpful to the operator in controlling the apparatus.

In calibrating these thermometers the same copper wire was used to connect them together which was subsequently used to connect them together in the calorimeter so that its coefficient has been taken into consideration. The thermometer leads outside the calorimeter have been connected in such a manner as to eliminate the effect of change in the resistance of the leads with varying temperatures. The resistance change in these thermometers and in the rectal thermometer used in measuring the change in temperature of the subject of experiment is read on a Kohlrausch rotary bridge.<sup>11</sup> The slide wire of this bridge has been especially adjusted so that no departures from uniformity in resistance exist that will affect the bridge readings by an amount which, translated into terms of temperature, would correspond to an error of 0.01°.

<sup>11</sup> F. Kohlrausch *Ann d Physik* (Wiedemann's Folge), lvi, p. 177, 1895.

For connecting the three thermometers successively to the bridge a special precision rotary switch with silver contacts has been used. The variations in the contact resistance of this switch are too small to affect the accuracy of the thermometric measurements to a significant extent. The galvanometer used in connection with this bridge and also for making readings of the condition of the thermocouple systems is an instrument of the D'Arsonval type, constructed by Siemens and Halske. The resistance of the moving system is 50 ohms and a ballast resistance of 150 ohms built into the instrument has always been used in series with the system to prevent excessive damping and secure rapid readings. A thermo-electric effect on the galvanometer is noticeable with change in room temperature. Readings were so made as to eliminate this.

A ballast resistance of 100 ohms has been introduced into the bridge battery circuit to prevent the possibility of changing the resistance of the thermometers by heating from the bridge current. The galvanometer is amply sensitive to give deflections for a lack of balance corresponding to  $0.005^{\circ}\text{C}$  with this ballast in the battery circuit. No heating effect in the thermometers is perceptible on keeping the bridge circuit closed as long as half a minute though this is never done in practice. The key is always kept closed sufficiently long to make sure of the reading and usually the final balance is made by rotating the hood of the bridge with the key held closed. The smallest graduations on the hood of the bridge correspond very nearly to  $0.01^{\circ}$  and temperatures could easily be read to  $0.001^{\circ}$ , but such readings would not be reliable. All the bridge readings are translated into terms of temperature by reference to curves.

To enable the operator to control the temperature of the water used to bring away heat from the interior of the chamber, the water is allowed to flow at a temperature several degrees cooler than it is estimated will be necessary and just before entering the chamber it flows over a coil of copper pipe containing an electrical heating resistance. By manipulation of a ballast resistance in series with this, the operator can adjust the heating effect and so control the rate of removal of heat from the box. As originally constructed, the heating resistance was fed directly with current from the lighting system of the building, and fluctuations in the voltage of this current together with variations in the temperature of the cold

water as it flowed to the heater, caused continual fluctuations in the temperature of the inflowing water. It was early recognized that these fluctuations might lead to errors in the measurement of small quantities of heat and an attempt was made to minimize the evil by diminishing the capacity of the water coil within the calorimeter so that the coil would be emptied two or three times during the four-minute intervals between the readings of the water thermometers. The coil (fig 2) consists of two large inflow and outflow tubes, *A* and *B*, connected by a series of fine copper tubes *C*. In length and width it is nearly equal to the corresponding

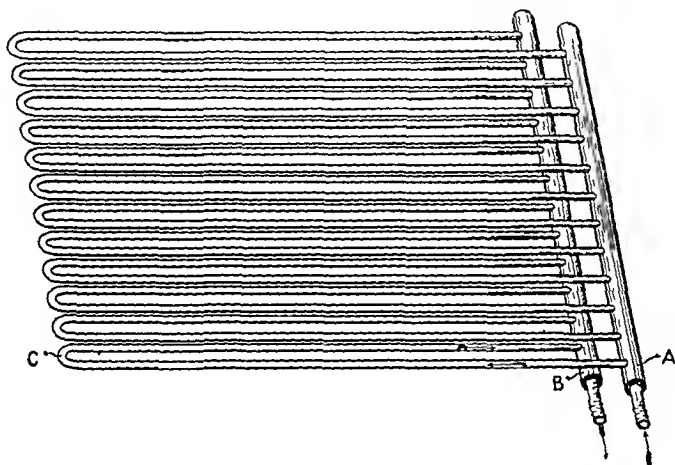


FIG 2 WATER COIL

dimensions of the copper chamber and is suspended from the top of the chamber. Just before the water leaves the chamber it passes through a small mixer (not shown).

It was found that even after diminishing the capacity of the water coil fluctuations in the temperature of the entering water were not paralleled by corresponding fluctuations in temperature of the outflowing water. Control experiments on the measurement of small amounts of heat made at this time indicated that the results were not trustworthy. The difficulty was finally overcome by feeding the water to the heater at a constant temperature and providing for control of the fluctuations in the heating current.



The first condition was secured by allowing the water to flow through about twelve meters of copper tubing coiled in a tank of water provided with a Gouy temperature regulator<sup>12</sup> similar to that employed by Barnes<sup>13</sup> in his calorimetric work on the specific heat of water. This device insures great constancy in the temperature of the water as it leaves the long coil of copper tubing, the fluctuations being of the order of  $0.01^{\circ}\text{C}$  either side of the mean. After leaving the copper coil of the Gouy regulator tank, the water is brought to the heater through a vacuum jacketed glass tube. For controlling the fluctuations in the heating current the device represented in figure 3 was used.

It consists of a glass bulb with an inner tube reaching nearly to the bottom and dipping beneath the surface of a quantity of mercury at the bottom. Above the mercury the bulb is filled with chloroform. Surrounding the inner tube and immersed in the chloroform, within the bulb, is a coil of manganin resistance wire the resistance of which is about one-tenth that of the main heating resistance. The ends of this coil are fused to platinum wires which lead out through horns of lead glass at the top.

The stopcock at the bottom is for convenience in filling and has no other function. At the top the inner tube emerges and terminates in a three-way stopcock above which is a capillary continuation of the inner tube. Just below the stopcock a fine platinum wire is fused into the side of the tube. The heating resistance and the entire bulb of the regulator are enclosed in a brass container through which the calorimeter water flows.

The upper end of this brass box is closed with a stopper through which the stem of the apparatus passes. Two short pieces of rubber tubing are tied over the glass horns through which the wires from within the bulb emerge. The upper ends of these pieces of rubber tubing are slipped tightly over the ends of two pieces of glass tubing which pass through the stopper of the brass outer case. In this manner the wires can be led out securely insulated from the water. The coil within the bulb is connected in series

<sup>12</sup> Gouy *Journ d physique*, vi, p 479, 1897

<sup>13</sup> Barnes. On the Capacity for Heat of Water between the Freezing and Boiling Points together with a Determination of the Mechanical Equivalent of Heat in Terms of the International Electrical Units, *Phil Trans Roy Soc*, London, Series A, clcix, pp 149-263, 1902

with the main heating resistance as shown in the figure. By turning the three-way stopcock, the mercury in the bulb can be connected with the capillary tube at the top or with a small reservoir of mercury at the side. When the current is first turned on the heater, the cock is always placed so as to connect the mercury in the bulb with the reservoir. After the temperature of the

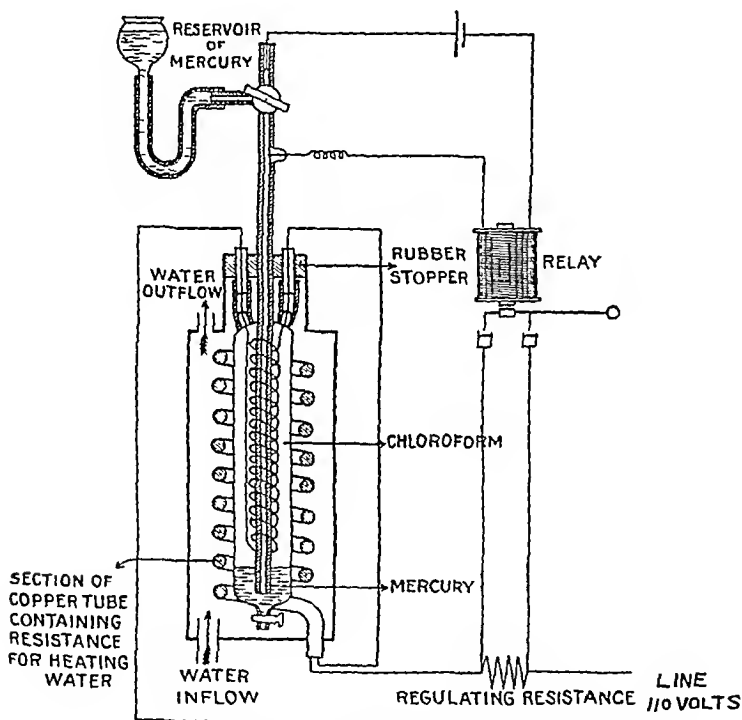


FIG 3 WATER HEATING RESISTANCE AND CURRENT REGULATOR

incoming water has reached approximately the correct level, the cock is turned so as to put the capillary tube in connection with the mercury in the bulb. A little more current is then turned on the heater and the immediate effect is to cause an expansion of the chloroform by reason of the sudden increase in the heat developed in the manganin wire within the bulb. The expansion of the chloroform drives mercury up in the capillary tube and closes

the relay circuit The operation of the relay opens a short circuit around which the main heating current has been flowing and forces the current to pass through a resistance marked "regulating resistance" in the figure This causes a diminution in the strength of current, the volume of the chloroform shrinks, the relay circuit is broken and then the cycle is repeated The low specific heat and high coefficient of cubical expansion of chloroform result in very rapid changes in its volume with change in the amount of heat developed in the wire within the bulb, and as long as the line voltage remains constant these changes succeed each other with great regularity and the mean heating effect of the current flowing through regulator and heater is constant

A fall in line voltage results in a lessening of the mean volume of the chloroform which causes the time of closure of the short circuit to become greater and thus compensates for the fall in voltage by lengthening the time during which the resistance of the circuit is a minimum A rise in voltage results in increase of the mean volume of the chloroform with the attendant effect of keeping the regulating resistance in series with the heater for a greater length of time and thus compensating for the increase in current which a rise in line voltage would otherwise produce

The chloroform responds so quickly to changes in the heating effect of the current that regulation takes place before any change in the temperature of the flowing water becomes appreciable As in the Gouy regulator, the platinum contact wire at the top of the capillary tube is kept agitated mechanically to prevent "sticking" of the mercury

The original regulator is still in use and has caused no trouble over a period of two academic years At its first trial it maintained the temperature of the water constant within  $0.05^{\circ}$  over a period of five hours Three-fifths of this change was traced to displacement of the upper contact and over the last four hours of the experiment the change was only  $0.02^{\circ}$  During the course of actual experimental work changes of more than  $0.01^{\circ}$ – $0.02^{\circ}$  practically never occur except when it becomes necessary to change the temperature voluntarily

When this occurs the change is made at the time of reading the thermometers so that an interval of four minutes elapses during which conditions may become steady Small changes in tem-

perature can be brought about by turning the three-way stopcock so as to add or remove small amounts of mercury from the capillary tube

For greater changes the bulb is connected to the reservoir and the ballast resistance used as at the beginning of an experiment

Immediately after making the alterations described above, the control experiments on the measurement of small quantities of heat became satisfactory

On account of the slow rate of flow and the small diameter of the tubes through which the calorimeter water passes, it was decided not to use the city service water which always deposits a great deal of sediment, but to use distilled water. The supply is kept in a large storage tank, *A*, figure 4, and is raised by a small bronze geared pump to the tank, *B*, figure 5. Tank *B* is provided with an overflow pipe and the water is pumped up rather faster than it is needed so that a little always comes back through the overflow pipe and the head of the water remains constant.

From *B* separate pipes bring down the water for cooling the interior and for cooling the air space between the outer and middle walls. The water for cooling the interior passes down from *B* through a long coil of copper tubing in the tank *C*, figure 4. This tank is heavily lagged with cork and is filled with broken ice. Leaving tank *C* at a very low temperature, the water passes through the coil of copper tubing in the Gouy regulator tank, *D*, figure 5. Leaving this tank at constant temperature, usually about  $16^{\circ}\text{C}$ , it flows through the vacuum jacketed tube *F*, figure 5, to the apparatus *R*, shown in the same figure. *R* contains the heating coil and the device shown schematically in figure 3 for controlling the heating current. From *R* the water enters the calorimeter. The entrance and exit of the water is through vacuum jacketed glass tubes in which are placed the bent mercurial thermometers for determining the temperature of the ingoing and outcoming water. The vacuum jackets have been thoroughly exhausted with a Gaede mercury pump, the tubes being heated to a high temperature during exhaustion to drive off occluded gases from the walls. The vacuum is of the order of that in a cathode tube and the very perfect heat insulation afforded effectively protects the thermometers and water current against the influence of the higher temperature of the walls and air spaces through which

they pass After passing out of the calorimeter the water flows over and is collected in the can *E*, shown in figure 4 suspended from the balance At the end of an experimental period, while the water in this can is being weighed, the water flowing from the calorimeter is allowed to collect in the copper pitcher shown at *P*, figure 4

When the weighing is completed the contents of the pitcher are emptied into *E* after deflecting the water current to this can once more The jointed pipe which permits the deflection of the water current can be seen just above *P* in the figure It is shown feeding into a waste pipe which leads back to the storage tank *A* During the preliminary periods when heat is not being measured the water is allowed to take this path At the bottom of *E* can be seen a cock through which the can may be emptied if in the course of a long experiment it becomes too full

The water for cooling the air space between the outer and middle walls is refrigerated in a tank similar to *C* which is not shown in figure 4 as it is concealed behind the calorimeter The return flow from this system is allowed to run directly back into *A* Below *C* can be seen a waste pipe for draining off the water from melting ice In figure 4, the oxygen cylinder *O* is shown at the side of the calorimeter and on top of the calorimeter the magnet *M*, which opens and closes the oxygen inflow tube At the left of the magnet is the large tambour or tension equalizer with pointer and scale

At the left of figure 5 is shown the table on which is assembled the physical apparatus for determining the temperature of the air and walls and that of the subject of the experiment, also the various devices for maintaining the adiabatic control

In the center of the table at *Br* is the Kohlrausch rotary bridge At the left of this is the thermometer switch marked *T S* A tapping key between the thermometer switch and the bridge is used to close the bridge and battery circuits Behind the tapping key is a switch for connecting the galvanometer either with the bridge or with the thermo-couple system This switch is provided with a pair of contacts for short circuiting the galvanometer At the right of the bridge is a second tapping key in the thermo-couple circuit and at the extreme right a rotary switch by means of which the various groups of thermo-couples can be connected with the galvanometer

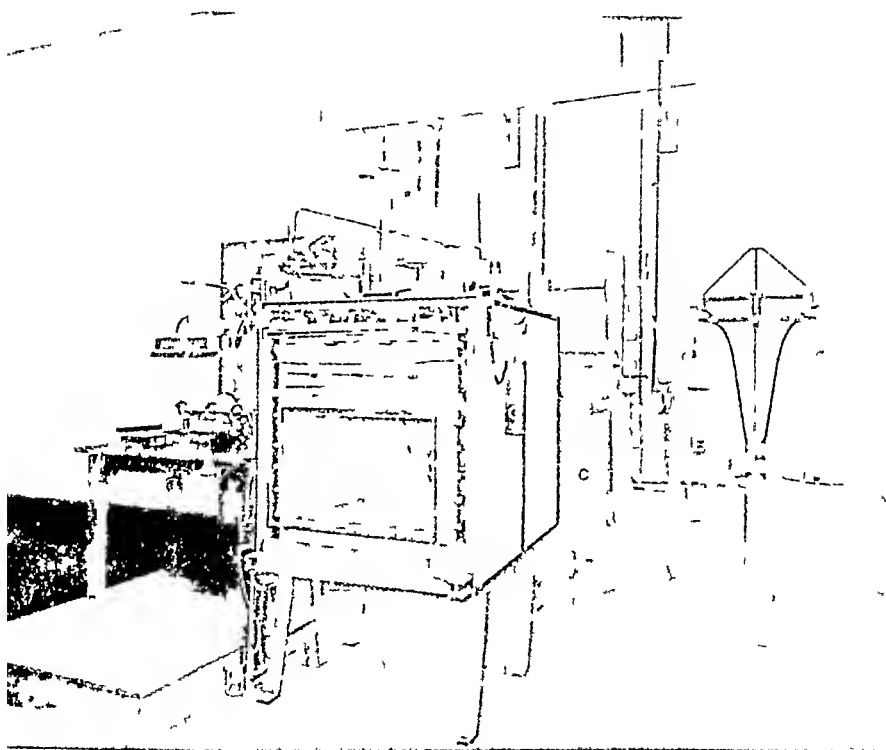


FIG 4 VIEW OF CALORIMETER SHOWING PART OF WATER SYSTEM



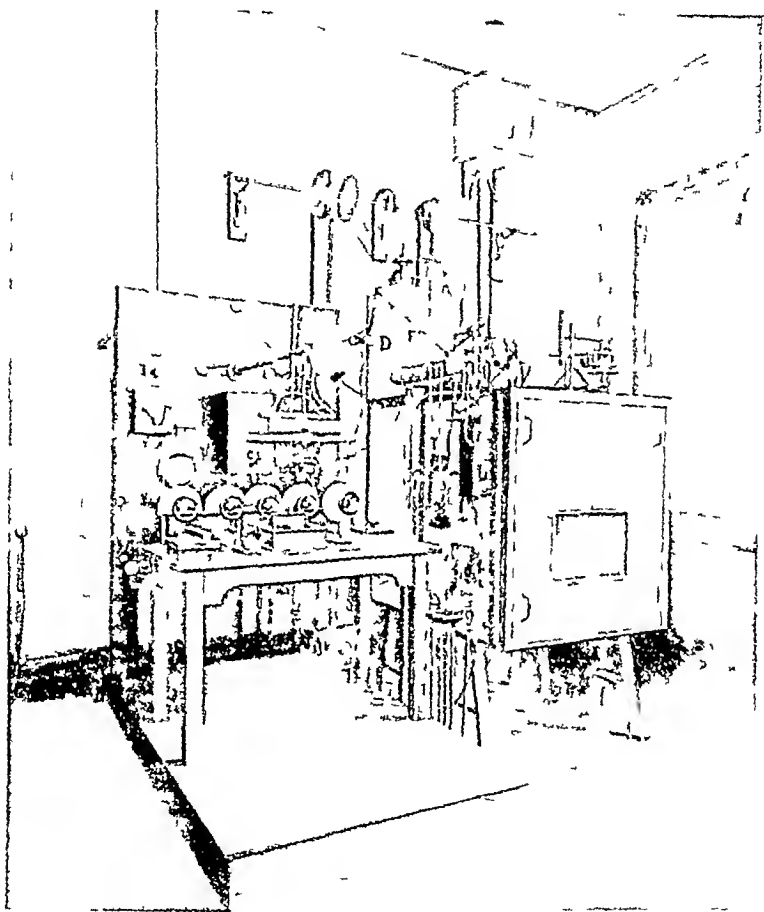


FIG 5 VIEW OF TABLE FOR PHYSICAL MEASUREMENTS AND CONTROL





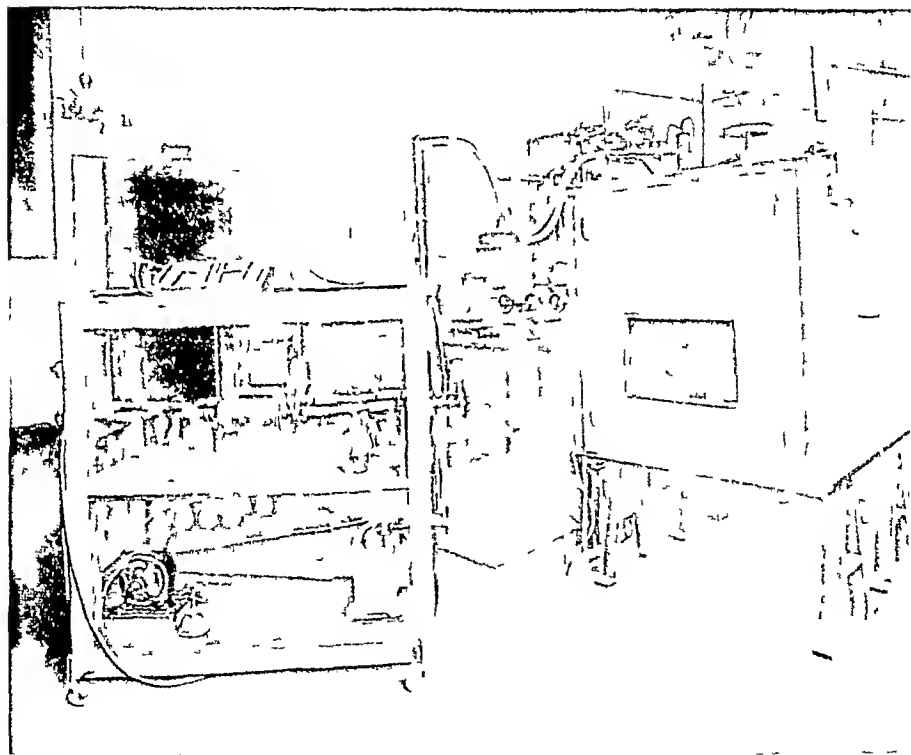


FIG 6 ABSORBER TABLE AND APPARATUS FOR ANALYSIS OF RESIDUAL AIR



The galvanometer stands on a shelf under the box marked *G*. It is read with a telescope and scale shown in the figure with a lamp which serves to illuminate the scale. The ballast resistances for controlling the current in the heating wires in the outer air space are mounted on the back of the large slate switchboard shown behind the table. They are manipulated by the handwheels at the back of the table. The rheostat for controlling the current in the water heating resistance is also mounted behind this board and is operated by the handwheel at the extreme right. This rheostat has sixty steps. The mounting of the resistances on a switchboard in this manner was adopted primarily because it was necessary to construct the apparatus in such a manner that it would conform to the requirements of the board of fire underwriters. The plan has several distinct advantages. The heat from these resistances, which is considerable, is kept away from the apparatus on the table, and since the handles of the rheostats are thoroughly insulated, no part of the circuit carrying high potential current for heating is connected in any way with the table. When it is remembered that the apparatus on the table is used in connection with a delicate galvanometer the last consideration will be seen to be important. At the right of the table the mercurial thermometers are shown at *T*, figure 5, and below and behind them is a small board of hard rubber on which terminate the wires leading from the thermometer and thermo-couple systems.

Below *R* is seen a small switchboard of slate on which terminate the ends of the 110 volt heating circuits. Throughout the construction no pains were spared to secure thorough insulation and substantial fastening of the wiring and no trouble has been experienced from this source in two years of experimental work. At the right of the galvanometer on the large switchboard the relays for the Gouy regulator and for the regulator in *R* are shown rather indistinctly.

In figure 6 the absorber table is shown. The arrangement of motor, pump, sulphuric acid wash bottles and soda-lime cans, *S L*, is clearly shown. Above can be seen the rubber tubes which lead the air from the calorimeter to the absorber table and carry the purified air back to the chamber. On the top of the table are the gas meter and U-tubes for determination of the composition of the residual air at the ends of the experimental periods.

Fluctuations in barometric pressure are measured on a standard barometer of the Fortin type which is not shown in any of the figures

#### CONTROL EXPERIMENTS

Control experiments of four types have been carried out to determine the magnitude of the errors which may be expected to occur in the work. These experiments may be designated Radiation and conduction tests, Electric controls, Alcohol controls, Oxygen blanks

##### *Radiation and conduction tests*

The radiation and conduction test is made for the purpose of determining how far the adiabatic wall prevents gain or loss of heat, in other words, whether under experimental conditions the walls are really adiabatic. In making this test no source of heat is put into the box and no cooling water flows through the interior system of piping. The box is closed up and the temperature of the zinc wall adjusted to be the same as that of the copper wall and so maintained. The air and wall thermometers are then read at frequent intervals.

The conditions and result of one such experiment were as follows

Experiment began at 9 00 a. m. At the start the temperature of the laboratory air was  $21^{\circ}$ . The temperature of the wall of the copper chamber was  $23.20^{\circ}$  and that of the air within the chamber was  $23.50^{\circ}$ . At 11 20 a. m. the temperature of the wall had fallen to  $23.19^{\circ}$  and that of the air inside to  $23.495^{\circ}$ . The hydrothermal equivalent of this calorimeter is 10.75, or, otherwise stated, a change of  $1^{\circ}\text{C}$  in the temperature of the copper wall under experimental conditions, means the absorption or setting free of 10.75 calories. A fall in temperature of the copper wall of  $0.01^{\circ}$ , the result of loss of heat from the calorimeter to the outside, would result in an error of 0.1 caloric. Such a loss occurred in this experiment in the course of two hours and twenty minutes, or a period equal to two and one-third times the length of the usual experimental period. This loss, however, occurred under conditions far more severe than those of actual experiment as the air of the laboratory was purposely kept over three degrees cooler than that within the chamber. In actual experimentation the air of the laboratory is always kept as nearly as possible at the same temperature as that within the box and seldom differs from it by more than  $0.5\text{--}1^{\circ}$ . With this flatter gradient the loss would undoubtedly be very much less.

It appears then that with steady conditions the walls may be considered adiabatic without liability of incurring errors as great as 0.05 calorie per hour. Few of the experiments have lasted longer than three or four hours so that the chance for accumulation of the radiation and conduction error does not enter into the consideration. With experiments of long duration it might be worth while to attempt a careful determination of the radiation-conduction constant under experimental conditions and correct for it.

### *Electric control experiments*

The most precise and convenient method for controlling the accuracy of a heat-measuring apparatus is that of dissipating within the apparatus in the form of heat a carefully measured amount of electrical energy, measuring this heat with the apparatus and determining how closely the measured heat agrees with the amount which the electrical measurements show to have been dissipated during the time of experiment.

To secure uniformity in the electrical current and therefore in the amount of heat dissipated, an accumulator battery has been used as the source of current. This battery is of sufficiently large capacity (about 45 ampere-hours) to deliver the required amount of energy over periods of four or five hours without much diminution in voltage. The gradual fall in voltage can be easily and exactly compensated by manipulation of a ballast resistance. The scheme of the connections is shown in figure 7.

All of the resistances employed are made of material whose resistance changes but little with temperature. The current passes from the battery through a ballast resistance, then through the heating coil and back through a standard resistance. A precision millivoltmeter measures the fall of potential across the terminals of the standard resistance and serves to determine the current. From the terminals of the heating coil within the chamber a pair of wires runs out to a voltmeter. A key is provided in this circuit so that the voltmeter may be connected momentarily and measure the fall of potential across the terminals of the heating coil. The reading of the millivoltmeter is maintained constant by manipulation of the ballast resistance and the voltmeter is read several times during each period of the experiment. The voltmeter has

a range of 15 volts and is graduated in tenths of a volt. One hundredth of a volt can be easily estimated. With a fall of potential of the order of 5 or 6 volts the reading can be made to about one part in five hundred. The current can be measured with the millivoltmeter to about the same order of accuracy in so far as concerns the reading error.

These instruments were specially calibrated for us by the makers, the Weston Electrical Instrument Company. The accuracy of this type of instrument and the permanency of the calibration with careful use is so well known as to require no further comment.

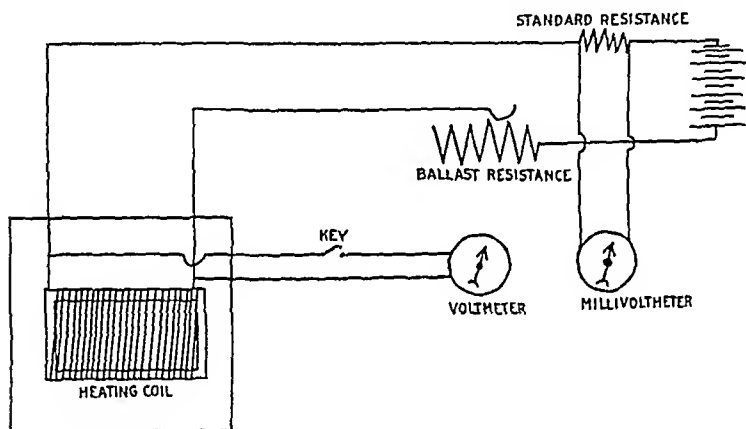


FIG 7 SCHEME OF CONNECTIONS FOR ELECTRIC CONTROL

Greater accuracy in the readings could doubtless have been attained by the use of a standard cell and potentiometer with a delicate galvanometer as indicator, but the errors introduced by the use of the direct-reading instruments are so small in comparison with other experimental errors that it has not been deemed worth while to further complicate the procedure.

The heat dissipated is given by multiplying together the numbers expressing the fall of potential across the terminals of the heating coil (in international volts), the current in amperes and the time in seconds and dividing by the number expressing the mechanical equivalent of heat at the temperature of the flowing water.

In our animal experimentation with the calorimeter up to the present time the mean temperature of the cooling water has been uniformly in the neighborhood of 20° and it has been kept in this region during the electric and alcohol controls. The value of the thermal capacity of water in joules per calorie for a large number of temperatures ranging from freezing to boiling has been determined by Barnes<sup>14</sup> in a series of extremely careful and painstaking experiments. The results of his experiments in the neighborhood of 20° agree very well with the corrected value obtained for this temperature by Rowlands using another method.

The difference is not sufficiently great to affect our results much whichever value is taken, but as Barnes' results were obtained by an electrical method and refer to the same electrical units as ours, it has been thought best to adopt his figure. This figure for 20° is 4.1873. To facilitate calculation instead of dividing the number of watt-seconds by this number we may multiply by its reciprocal, 0.2393.

The results of an electrical control experiment performed May 6, 1911 are given below.

The strength of current, *I*, was 2.1 amperes. The fall of potential across the terminals of the heating coil was 5.79 volts and the time for each period was 3600 seconds.

The heat is given by the product  $E \cdot I \cdot t \times 0.2393 = 10,470$ . This is expressed in small calories and is equal to 10.47 large calories.

The following is a tabulation of the results of this experiment.

HOURLY	CALORIES CALCULATED	CALORIES FOUND	ERROR
1	10.47	10.64	0.17
2	10.47	10.55	0.08
3	10.47	10.64	0.17

The uniformity of the conditions during this experiment can best be appreciated by an inspection of the readings of the water thermometers. The temperature of the inflowing water at the beginning of the third period was 20.56°C. There were fifteen readings during the period and for the first six successive readings, covering a period of twenty-four minutes, the temperature of the inflowing

<sup>14</sup> *Loc. cit.*



water was as given above. For the next six readings, another period of twenty-four minutes, the temperature was  $20.55^{\circ}$ . Then follow at four-minute intervals two readings of  $20.54^{\circ}$  and after the final four minutes the last reading is  $20.55^{\circ}$ . Whether these small variations are due to faulty functioning of the regulator *R*, we have never taken the trouble to ascertain. Slow changes of the order of  $0.01^{\circ}$  do not affect the accuracy of the results in measurable amount. It seems probable that the regulation could be made even closer if one felt the necessity of doing so. The temperature differences between the in and outflowing water during the same period are instructive as showing the uniformity of the dissipation and removal of heat. The first three differences were  $1.02^{\circ}$  and the remaining twelve  $1.01^{\circ}$ . One might be led to think that the first three differences were too high by  $0.01^{\circ}$ , but reference to the preceding period affords an explanation of this result.

During the second hour the temperature of the calorimeter fell slightly, but continuously, and this change was nearly at an end at the beginning of the third hour. The heat given up by the wall of the apparatus is responsible for the greater value of the first three temperature differences in the third period.

#### *Alcohol control experiments*

By the combustion within the chamber of a known quantity of alcohol, one is able to control the determination of water, carbon dioxide and oxygen as well as that of heat. In planning experiments to ascertain the magnitude of the errors which are liable to occur in using the apparatus with animals, it is of importance to provide that the amounts of alcohol consumed per hour shall be such as to dissipate about the same amount of heat as the animal may be expected to eliminate in the same time. With a given apparatus the experimental errors will be, assuming a uniform technique, about constant in absolute amount, so that the percentage error will diminish as the total quantity measured increases. In planning the calorimeter, one of the problems in view was the determination of the metabolism of very young infants, whose heat production might be expected to range between 10 and 20 calories per hour. It was determined on this account to attempt to burn alcohol in corresponding amounts in the control experiments.

In order to secure results of value in control experiments of this character it is important to secure complete combustion of the alcohol and so to regulate the supply that the combustion proceeds with the greatest possible uniformity

The method used was in general similar to that in use at the nutrition laboratory at Boston and is a modification of a method described by Atwater and Benedict<sup>15</sup> The alcohol lamp within the calorimeter was fed continuously with alcohol, from without, through a fine tube This tube ended outside in a narrow burette graduated in 0.01 cc Into this burette alcohol was allowed to drop from a supply bottle at a rate just equal to that of combustion That the rate of dropping was correct was indicated by the level of the alcohol in the burette which remained constant At the beginning of an experiment the lamp was lighted and the apparatus sealed After a preliminary period of half an hour or more, during which the calorimeter was brought into equilibrium, the burette was read and the supply bottle changed for another which had been weighed By means of a small stopcock on the tube leading from the supply bottle, the rate of flow could be quickly adjusted so as to permit of maintaining the level of alcohol in the burette constant to within 0.01–0.02 cc At the end of a period the burette was watched and the level kept as nearly as possible the same as at the beginning Any variation could be noted and correction made At first the attempt was made to make use of supply bottles on the Mariotte principle to secure a uniform head of liquid, but this was abandoned, as with the very slow rate of flow slight changes in room temperature made more change in the rate of flow than the changing head It proved more satisfactory to use bottles with a simple siphon and regulate the flow by hand with the stopcock as might be necessary

In this manner it was possible to secure very uniform burning of the alcohol To prevent incomplete combustion of the alcohol it was found necessary to use a lamp so constructed that the region of the edge of the wick should always be sufficiently hot to insure immediate ignition In the first experiments where larger amounts of alcohol (corresponding to about 35 calories) were burned, a lamp similar in construction to an Argand burner, with provision

<sup>15</sup> *Loc cit*

for access of air to the interior of the flame, was used with success. With the smaller amounts of alcohol the flame was so small that the metal edge of the lamp was cooled by conduction of heat away from it to the base and combustion was imperfect.

This became manifest by the lack of correspondence between results found and calculated and by a strong peculiar odor perceptible immediately after opening the chamber, presumably due to the presence of aldehyde and perhaps other products of incomplete combustion. By using a short piece of hard glass tubing for the top of the burner and a wick of glass wool, this difficulty was entirely overcome.

The alcohol used had a strength of 92.65 per cent as calculated from a determination of the specific gravity. The specific gravity was determined by weighing in a Squibb pycnometer equal volumes of boiled distilled water and of the alcohol in question, the measurement of the specimens in the pycnometer being carried out at the same temperature. The percentage was then determined by interpolation from the Squibb alcoholimetric tables.

For a discussion of the accuracy of this method reference may be made to the paper of Atwater and Rosa<sup>16</sup> previously cited.

The theoretical amount of oxygen required to burn a given weight of alcohol and the corresponding amounts of water and carbon dioxide which would result from its combustion, can be readily calculated from the chemical equation expressing the reaction which occurs when alcohol burns. In the case of the water one must make a correction for the amount of water of dilution present in 92.65 per cent alcohol. The heat of combustion of alcohol has been the subject of a large number of calorimetric investigations. As a result of twenty-five observations with the bomb calorimeter, Atwater and Rosa<sup>17</sup> found the heat of combustion of pure ethyl hydrate to be 7.067 large calories per gram. This figure has been adopted as the basis of the heat calculation in the present experiments. Below are presented the results of two alcohol control experiments. The first is a period of one hour during which 1.74 grams of alcohol were burned in the calorimeter, the smallest quantity we have attempted to burn up to the time of writing.

<sup>16</sup> *Loc cit*

<sup>17</sup> *Loc cit*

The second experiment performed two days later covers three periods of one hour each

*Alcohol control, May 13, 1911*

ALCOHOL	CARBON DIOXIDE		OXYGEN		WATER		HEAT	
	Found	Calc	Found	Calc	Found	Calc	Found	Calc
grams	grams	grams	grams	grams	grams	grams	calories	calories
1 74	3 10	3 09	3 37	3 37	2 35	2 02	11 70	11 42

*Alcohol control May 15, 1911*

2 605	4 70	4 62	5 12	5 04	3 47	3 02	16 80	17 09
2 420	4 40	4 30	4 70	4 68	3 11	2 81	15 56	15 88
2 390	4 28	4 24	4 73	4 62	3 06	2 77	15 29	15 65
	13 38	13 16	14 55	14 34	9 64	8 60	47 65	48 62

Inspection of this table shows that the results found agree fairly well with those calculated except in the case of water vaporized

The water found is seen to exceed that calculated by from 10 to 16 per cent. In the second experiment it may be further noticed that the percentage discrepancy steadily decreased throughout the experiment, being 14.9 per cent in the first hour and 10.4 per cent in the third. The explanation of this discrepancy is doubtless to be sought in the fact that the walls of the copper chamber are able to occlude on their surfaces considerable quantities of moisture and in addition, the material of which the air thermometers are constructed and the insulation of the wiring within the chamber are to some extent hygroscopic. The laboratory is situated within a short distance of a large body of salt water, the East River, and the air, never very dry, is sometimes nearly saturated with water vapor. This air has free access to the interior of the apparatus except when it is sealed up for experimentation. During the course of an experiment in which the amount of water given off within the calorimeter is small, the absorbing apparatus is capable of bringing about a continuous reduction of the vapor tension within the chamber. The greater part of the moisture present in the air of the chamber before beginning an experiment will be removed during the preliminary period, but as

the tension of water vapor in the air becomes less the moisture which has been occluded on the walls seems to be given off gradually. Doubtless, in an experiment of long duration, the water measured during the later hours would gradually approximate the amount generated by combustion in the same time. Similar results as regards the water determination have been noted by Benedict and Carpenter<sup>18</sup>. With an apparatus in which the amount of oxygen consumed can be directly measured, the amount of water given off by the subject becomes a matter of less importance than was the case in the older forms of respiration apparatus. The water excretion has not been a factor of importance in any of the physiological work for which the calorimeter has been used up to the present time. It is, however, a matter of great importance to know just how much water has evaporated and left the chamber during an experimental period. Heat is absorbed in the process of vaporization and unless correction be made for the heat rendered latent by vaporization and removed in the ventilating air current, the amount of heat measured will be too small. To determine the quantity of heat which has been rendered latent by the evaporation of a given weight of water, one must know the latent heat of evaporation at the temperature of the air within the calorimeter. This temperature in our experiments has been 26°C. Regnault's formula,<sup>19</sup>  $L = 606.5 - 0.695t$ , gives as the latent heat 0.588 large calorie per gram of water evaporated at 26°. This figure was used at the beginning of our work. It was adopted at that time provisionally until opportunity should offer to decide as to the best value of  $L$  for this temperature. Through an inadvertence the use of this factor has been continued up to the time of this publication and a large amount of experimental work has been calculated on this basis.

The most recent work on the heat of vaporization of water over the range of temperature in question appears to be that of Smith.<sup>20</sup> According to the formula developed by him the value of  $L$  at 26° would be in the neighborhood of 0.582 calorie per gram. The amount of water vaporized in our experiments is usually so small

<sup>18</sup> *Loc cit*

<sup>19</sup> Regnault *Mem Acad Roy Sci Inst France*, xvi, pp 635-728, 1847

<sup>20</sup> Smith *Heat of Evaporation of Water Physical Review*, xxv, p 145,

that it really makes but little difference which figure is used. The error would usually be in the second decimal place. In future work a new value for  $L$  will be used based on the best data available and, in a critical consideration of such experiments as have been done prior to July 1912, it may be well to recalculate those in which the evaporation of water has been unusually large, though the correction seems hardly likely to change the physiological significance of any of this work.

In the case of carbon dioxide the percentage error is seen to range from 0.3 per cent to 2.3 per cent and in the case of oxygen from zero to 2.3 per cent. In experiments with animals it is doubtful whether the results can be relied upon in the case of oxygen and carbon dioxide to better than 2 per cent when the total quantities measured are as small as in these control experiments. The absolute error as may be seen is not very great and if the results of a number of hours of experimentation be added together the total result would be much nearer the truth. In the second experiment the percentage error in the case of carbon dioxide for the three hours was 1.6 and that of the oxygen determination over the same time 1.5. Turning to the heat measurement, we find errors in the single hours of the order of 2 to 2.5 per cent and an average error for the three hours of about 2 per cent. When one considers the complexity of an apparatus for measuring at the same time heat, oxygen absorption and output of carbon dioxide and water and bears in mind the difficulty of calorimetric work in general, it will be appreciated that a percentage error as high as 2.5 on a total of 15 calories is not surprising. It seems hardly likely that the absolute error involved can prove of any consequence in the decision of the class of physiological problems involving this kind of measurement. From the results of a large number of electric control experiments in addition to the alcohol controls it seems reasonably certain that under the conditions of the controls the measurement of heat is trustworthy to 2.5 per cent. Over longer periods or in measuring larger amounts the percentage error is naturally less. That the same degree of accuracy can always be attained in experiments on living animals is open to question. In such experiments one must take into consideration fluctuations in body temperature. As the apparatus stands at present this is attempted by measuring with great accuracy the rectal temperature

with an electrical resistance thermometer. When the fluctuations in body temperature are gradual the weight of evidence seems to indicate that the rectal temperature is a sufficiently good index of the general body temperature. When, however, the temperature of the body undergoes rapid change, we have evidence that the change in the rectal temperature does not run strictly parallel to the general change and under such circumstances the heat measured over a short period may be in error by a very much larger amount than 2.5 per cent. The possible limits of this error and whether or not means which have been proposed for eliminating or lessening it will be effective cannot be stated as yet. This source of error is probably minimal in all cases where the body temperature changes slowly, but it is well for the present to exercise reserve in judging the results of experiments in which the rectal thermometer has shown sudden and large fluctuations.

#### ERRORS IN DETERMINATION OF OXYGEN

In attempting to determine oxygen consumption directly with this type of apparatus, numerous sources of error require attention. Oxygen is admitted to the system from a weighed cylinder of the gas so as to maintain the apparent volume the same at the end as at the beginning of the experiment. The following causes can operate to change the apparent volume.

Consumption of oxygen by the subject of experiment

Removal of excess of water vapor by absorbers

Removal of carbon dioxide by the absorbers

Change in temperature of air within the chamber

Change in general barometric pressure

All of these causes are operative during experimentation and all, except actual consumption of oxygen by the subject, require that corrections be made. In addition there is the possibility of leakage in any part of the system. Should a loss of 1 liter of air from the system occur, the determination of carbon dioxide and water would suffer but little error. That of oxygen would be in error by over 1.4 grams. In an experiment on a small human infant such a leakage would cause an error in the neighborhood of 30 per cent.

The correction for change in temperature of the air within the

calorimeter is made on the basis of the indications of the air thermometers. These are six in number and so situated as to integrate fairly well the average temperature of the air. Owing to the necessity of keeping the central part of the chamber clear, the air thermometers had to be put near the walls so that warmer or cooler air from the central parts of the chamber has to reach the thermometers by convection. The ventilation is not sufficient to prevent localized portions of this air from acquiring for short intervals a temperature very different from the general temperature inside the box in case a sudden increase in heat elimination occurs, as the following experiment shows.

An electrical resistance was put in the calorimeter and the apparatus sealed up. After steady conditions were attained the air thermometers were read and heat suddenly developed in the resistance which occupied a position in the center of the box.

The box itself with the tension equalizer provided with a long pointer is a most sensitive thermobarometer. The immediate effect of a puff of heat in the center of the box was to cause a sudden elevation of the pointer on the tension equalizer of about 10 cm. After a considerable interval the air thermometers recorded the change. This shows clearly that sudden changes in temperature at the end of an experimental period would be quite sufficient to upset the oxygen determination. Whether such changes occur or not the operator is fortunately in a position to determine without regard to the indications of the air thermometers. No sudden change in temperature can escape detection if the long pointer attached to the tension equalizer is watched and this the operator always does at the end of the periods. No result is accepted where a sudden change in temperature of the air occurs at or near the end of the period.

Changes in barometric pressure can be read to 0.05 mm. The barometer is always adjusted a few minutes before the end of a period so that the exact reading can be obtained without delay just at the end of the period. Since the residual amounts of carbon dioxide and water vapor in the chamber may vary from the end of one period to that of the next an analysis is made just before the end of each period by the method described by Benedict and Carpenter<sup>21</sup>.

<sup>21</sup> *Loc cit*



Any error in the residual analysis would also appear in the final result as affecting more or less seriously the determination of oxygen

For the purpose of determining the adequacy of the technique and the tightness of the apparatus without the necessity of going through with the very considerable labor of an alcohol control, the following experiment was devised which for brevity has been called an

### *Oxygen blank*

In carrying out this test the apparatus is sealed up as if for a formal experiment and the ventilating current of air started. No source of heat within is necessary and the cooling water need not be kept flowing though this has sometimes been done. When conditions have become fairly steady an analysis of the residual air is made and the air pump stopped. The air current is deflected in the usual manner through a fresh set of weighed absorbers and the tension equalizer adjusted to standard position by admission of oxygen.

The air current is started again, the cylinder of oxygen weighed and replaced and the experiment continued for an hour when the residual analysis is again made and the tension equalizer adjusted to standard position. No oxygen will have entered the system, provided it is tight, unless a shrinkage in volume due to fall of temperature or rise of the barometer has occurred. To prevent an increase in apparent volume the temperature is made to fall slightly throughout by use of the cooling water in the outer air space. Just at the end of the preliminary period and just at the end of the test the barometer and air thermometer readings are made.

After correcting for the changes in volume due to temperature and barometric change and making allowance for the amounts of water vapor and carbon dioxide removed by the air current the net amount of oxygen used should be zero. In one such experiment the amount of oxygen unaccounted for after making the corrections was 20 cc and in another 66 cc. These represent the extremes. The amounts usually run about 20–30 cc. The smallest oxygen consumption in actual experiments has been with infants

The amount for the smallest infant (weight 3.05 kgms) was 2.16 to 2.50 grams per hour<sup>22</sup>

The average error shown in the oxygen blanks would be less than 2 per cent of this amount<sup>23</sup>. This blank experiment is easily performed and requires but one or at most two persons to carry it out. The ease with which it can be done makes it a convenient check on the gas analysis technique and it has been frequently employed. It will detect with certainty very small leaks. The window has to be sealed for each experiment and no satisfactory test for tightness can be made with the subject in the apparatus. The window is closed with two glasses, however, and sealed with great care. All but one of the joints on the absorber table can be tested during the experiments. All other possibilities of leakage are accounted for by the oxygen blank.

As a result of these tests and of the alcohol controls it seems to be established that the apparatus is capable of measuring oxygen in amounts corresponding to the metabolism of small animals to a very high degree of accuracy, but that this degree of accuracy shall be realized in actual experimentation on animals it is unconditionally essential that no sudden fluctuation in temperature of the air within the chamber shall occur at or near the end of the period of experimentation.

<sup>22</sup> Howland *Zeitschr f physiol Chem*, lxxiv, p 12, 1911

<sup>23</sup> The error in oxygen blanks was often less than 20 cc but the general trend of these experiments indicates an absolute error in oxygen measurement of about 20-30 cc. This for the smallest oxygen consumption measured would be an error of 1.25-1.5 per cent by weight.



# ANIMAL CALORIMETRY

## SECOND PAPER

### METABOLISM OF THE DOG FOLLOWING THE INGESTION OF MEAT IN LARGE QUANTITY

By H B WILLIAMS, J A RICKE AND GRAHAM LUSK

(From the Physiological Laboratory, Cornell Medical College, New York City)

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#### I INTRODUCTION

The construction of a respiration calorimeter which can measure the heat production, the carbonic acid outgo and the oxygen intake in hourly periods in the dog, has revealed some new facts which are to be set forth in this series of papers. The special object of much of the work done has been to investigate more closely the nature of the processes involved in the increased heat production which follows the ingestion of the various foodstuffs—the so-called "specific dynamic action" of Rubner. Carl Voit believed that abundant food increased the power of the cells of the body to metabolize the materials brought to them. Thus he clearly stated in the following language <sup>1</sup>

<sup>1</sup> Carl Voit *Physiologie des Stoffwechsels und der Ernährung*, 1881, pp 308, 311

In cases where the non-protein respiratory quotient rises above unity, the heat production may be approximately calculated by multiplying the number of liters of oxygen absorbed by its calorific value when the respiratory quotient is unity, and adding to that a certain value obtained as described below

The simplest expression for the conversion of dextrose into fat is as follows <sup>14</sup>

$$191.3 \text{ grams dextrose} = 100 \text{ grams fat} + 7.4 \text{ grams H}_2\text{O} + 84 \text{ grams O}_2, \text{ or} \\ 1 \text{ gram dextrose} - 0.43 \text{ gram O}_2 = 0.57 \text{ gram fat}$$

This reaction, however, does not probably take place with the liberation of free oxygen, but rather with the coincident metabolism of dextrose in a manner similar to that suggested by Bleibtreu<sup>15</sup> and represented by the following formula

$$270.06 \text{ grams dextrose} = 100 \text{ grams fat} + 54.6 \text{ grams H}_2\text{O} + 115.45 \text{ grams CO}_2$$

In this formula fat is produced from dextrose with the liberation of carbonic acid and water. The reaction is *exothermic*, 4.7 per cent of the energy in dextrose being liberated as heat as is seen below

$$270.06 \times 3.692 = 997.2 \text{ calories} \\ 100.00 \times 9.500 = 950.0 \text{ calories}$$

Since the elimination of 115.45 grams of carbon dioxide is accompanied by the liberation of 47.2 calories, it follows that 1 gram so eliminated has a caloric value of 0.409 or 1 liter, 0.803 calories. Recalling the fact that the R Q of carbohydrate is unity, one may deduct the number of liters of non-protein oxygen absorbed from the number of liters of non-protein carbonic acid eliminated and the remainder will represent the liters of carbonic acid due to the reaction involved in the production of fat from carbohydrate. Its calorific value is obtained by multiplication with 0.803 calories. At least this method of calculation may be provisionally adopted, pending a more complete knowledge of the process itself.

<sup>14</sup> Magnus-Levy *Von Noorden's Handbuch der Pathologie des Stoffwechsels*, 1, p. 166, 1906, Magnus-Levy and Meyer *Oppenheimer's Handbuch der Biochemie*, IV, p. 472, 1911

<sup>15</sup> Bleibtreu *Pflüger's Archiv*, LXXV, p. 345, 1901

CALORIES			BODY TEMPERATURE			MORNING WEIGHT IN KG	REMARKS
OG	Total Calc	Total Found	Start	End	Diff		
47	25 69	24 32	38 01	37 90	-0 09	13 74	Asleep
63	25 94	26 82	37 94	37 90	-0 04	13 50	Awake, some slight movements
42	21 54	22 78	37 86			13 80	Asleep
85	22 28	21 97	38 14	37 99	-0 15	15 78	Asleep
22	45 04	47 18	38 54	38 76	+0 22	13 90	Violent move- ments

of the dog is 0.6958 square meter, calculated from the formula  $11.2 \sqrt[3]{13.8}$ . The

		BODY TEMPERATURE			MORNING WEIGHT IN KG	C TO BODY	DEXTROSE TO BODY	O <sub>2</sub> CALC	O <sub>2</sub> FOUND
Calc	Total found	Start	End	Diff					
						gram	gram	gram	gram
03	37 38	38 14	38 69	+0 55	13 78				
97	32 86	37 74	38 44	+0 70	15 62				
35	33 61	37 77	38 48	+0 71	15 73				
20	38 01	37 98	38 38	+0 40	15 30				
70	38 91	38 44	38 45	+0 01	15 62	0 04	0 10	12 99	13 63
08	37 92	37 80	38 47	+0 67					
62	32 57	38 32	38 70	+0 38	13 85	0 79	2 00	12 48	12 57
77	31 60	37 64	38 02	+0 38	13 82	0 88	2 20	12 26	12 26
42	41 36	37 58	38 07	+0 49	13 78	0 46	1 01	13 48	14 43
40	41 29	39 31	39 55	+0 24	13 90	0 77	1 93	12 56	13 29
12	39 41	36 79	36 79			1 46	3 85	13 61	13 25
02	39 57	38 78	38 69	-0 09	13 59	1 09	2 75	13 02	14 28
67	38 26	38 69	38 46	-0 23		1 30	3 25	13 32	13 80
13	44 08	38 46	38 58	+0 12		0 74	1 86	14 27	15 17*
97	41 32	38 48	38 58	+0 10	15 62	1 15	2 88	13 00	12 80
06	41 16	38 58	38 51	-0 07		0 93	2 33	13 66	14 12
83	39 63	38 51	38 49	-0 02		1 06	2 65	13 30	13 43
17	36 12	38 38	38 34	-0 04	15 60	0 90	2 25	12 41	11 90
60	36 27	38 34	38 34			0 96	2 40	12 25	11 61
90	34 31	38 34	38 29	-0 05		1 14	2 85	11 75	11 96
27	30 66	38 33	38 29	-0 04	15 08	1 01	2 53	11 64	11 54
82	35 86	38 27	38 28	+0 01	15 84	1 37	3 42	12 13	11 34
54	31 29	38 24	38 30	+0 06		0 78	1 95	11 54	11 53
32	27 71	38 12	38 04	-0 08	16 07	1 10	2 75	9 65	9 31
26	28 99	38 04	38 13	+0 07		0 52	1 30	9 47	9 51
65	29 80	38 12	38 02	-0 10		0 97	2 43	10 01	9 43
73	31 44	38 02	38 15	+0 13		0 26	0 65	10 18	10 13
87	29 21	38 15	38 12	-0 03					
00	27 33	38 10	38 10		15 08				
53	27 98	38 10	38 08	-0 02					
03	24 34	38 08	38 01	-0 07					



## EXPERIMENTAL PART

Experiments with Dog I determined the effect of giving 1200 grams of meat and 700 grams of meat on alternate days at noon. This diet to which a little bone ash was added was maintained almost continuously from November 1, 1910 to May 1, 1911. The results with Dog II were obtained after giving 1000 grams of meat.

*A The effect of 1200 grams of meat ingested by Dog I*

The basal metabolism was determined during the hour between 9 45 and 10 45 a m on the day following the ingestion of 700 grams of meat at noon. Attempts made to obtain the metabolism of the dog between 10 45 and 11 45 were invariably frustrated on account of the activity of the dog previous to the regular meal time. The essentials are presented in Table I. The lowest metabolism was found to be about 22.3 calories per hour although this might rise to 25 calories even in the sleeping dog. It is possible that these differences in metabolism may have been due to variations in posture. Similar variations were also noted in Dog II. In Experiment 8, Dog I, when the metabolism was 26 calories per hour, the dog was quiet, awake, licked her paws and made some slight movements toward the end of the experiment. In Experiment 2 there were violent movements and the heat production was double the minimal basal metabolism, rising to 45 calories.

Twelve hundred grams of meat were given the dog at noon and the results of the various experiments are presented in detail in Table II. In order to bring out some of the more essential points, Table III has been constructed.

Consideration of this table suggests one point which must be further examined. To what extent is the nitrogen elimination during the period of an hour representative of the protein metabolism of the hour? Protein consists of a variety of amino-acids and it should cause no surprise if some of these were oxidized more readily than others. Besides this, there is certainly some retention of urea, as was shown by Hohlweg and Meyer<sup>16</sup> who noted a rise of urea in the blood from 0.038 per cent in fasting to 0.057 per cent seven hours after meat ingestion in the dog. Also, poly-

<sup>16</sup> Hohlweg and Meyer *Hofmeister's Beiträge*, vi, p. 397





TABLE IV  
Dog II—Urinary analysis after meat ingestion 500 grams of meat ingested at 11 00 a m

DATE	TIME	S	N g	TOTAL N	UREA + NH <sub>4</sub> -N		CREATINE N		CREATININE N		UNDETER- MINED N
					gram	per cent of total N	gram	per cent of total N	gram	per cent of total N	
1911 Dec 26	*9 00-11 00	0 014	12	0 1825	0 1656	90 5	0 0012	0 68	0 0004	0 22	8 60
	11 00-12 00	0 023	11	0 2555	0 2162	84 6	0 0020	0 78	0 0018	0 70	13 92
	12 00- 1 00	0 040	13	0 5082	0 4212	82 8	0 0156	3 10	0 0017	0 33	13 77
	1 00- 2 00	0 052	11	0 5748	0 4830	83 5	0 0148	2 50	0 0014	0 24	13 72
	2 00- 3 00	0 050	13	0 6290	0 5404	85 9	0 0130	2 10	0 0014	0 22	11 78
	3 00- 4 00	0 058	13	0 7525	0 6739	84 4	0 0119	1 50	0 0011	0 14	13 96
	11 00- 4 00	0 223	12 2	2 7200							

1000 grams meat ingested at 11 00 a m

DATE	TIME	S	N g	TOTAL N	UREA + NH <sub>4</sub> -N		CREATINE N		CREATININE N		UNDETER- MINED N
					gram	per cent of total N	gram	per cent of total N	gram	per cent of total N	
1912 Apr 29	11 00-12 00	0 0435	14 6	0 6360	0 5918	93 0	0 0331	5 2	0 0062	0 97	0 83
	12 00- 1 00	0 0669	12 8	0 8592	0 7539	87 7	0 0466	5 4	0 0063	0 73	6 17
	1 00- 2 00	0 0840	12 1	1 0193	0 9272	90 8			0 0033	0 32	
	2 00- 3 00	0 0856	12 5	1 0755	0 9463	87 9	0 0695	6 4	0 0023	0 21	5 49
	3 00- 4 00										
	4 00- 5 00	0 0949	13 0	1 2369	1 1457	92 6	0 0710	5 7	0 0023	0 19	1 51
	5 00- 6 00	0 0884	14 2	1 2608	1 1711	92 8	0 0647	5 1	0 0022	0 18	1 92
	11 00- 4 00	0 4623	13 4	6 087							

\* Results are given in grams per hour

peptid or amino-acid nitrogen may be retained in the organism if amino-acids be ingested. This was shown by Luthje<sup>17</sup> for glyco-coll and asparagine, by Murlin<sup>18</sup> for glyco-coll and by C G L Wolf<sup>19</sup> for alanine. The newer work of Folin<sup>20</sup> describes the retention of non-protein nitrogen which is not urea nitrogen in the muscle cells of a cat after glyco-coll has been given *per os*, the blood vessels to the kidneys having been previously tied off. The non-protein nitrogen also rose in the blood during the first hour after the ingestion of glyco-coll.

It is also known from the experiments of Rubner<sup>21</sup> on the dog, that the sulphur of protein is more readily eliminated than the nitrogen belonging to it. This is confirmed by the experiments of Wolf<sup>22</sup> as regards meat ingestion in man, being evident when veal cutlets were given but not evident when casein (plasmon) was administered. In the dog, however, Wolf found that the elimination of the two elements ran parallel. Since the N : S ratio in meat is 16 : 1, one would expect to find this ratio in the urine, or one a little higher, since some sulphur is present in the feces and some in the intestinal gas. The following experiments confirm Rubner's statement of an earlier elimination of sulphur than of nitrogen (see Table IV). It may be estimated that 22 per cent more sulphur was eliminated during the first six hours after the ingestion of 1000 grams of meat than corresponded to the nitrogen excreted during this period. This table also shows that by far the greater part of the urinary nitrogen eliminated during the early hours after meat ingestion consists in urea +  $\text{NH}_3$  nitrogen which is indicative of protein metabolism and not the elimination of nitrogen derived from the extractives.

In relation to the intermediary metabolism of protein it may be recalled that Reilly, Nolan and Lusk<sup>23</sup> found that after giving meat to phlorhizinized dogs the sugar formed from protein invariably appeared in the urine of the first few hours in greater amount than did the nitrogen of the protein from which it originated.

<sup>17</sup> Luthje *Congress für innere Medizin*, 1906, p. 44

<sup>18</sup> Murlin *Amer Journ of Physiol*, xii, p. 250, 1907

<sup>19</sup> Wolf *Biochem Zeitschr*, xl, p. 193, 1912, xli, p. 111

<sup>20</sup> Folin and Denis *this Journal*, xi, p. 87, 1912

<sup>21</sup> Rubner *Die Gesetze des Energieverbrauchs*, 1902, p. 368

<sup>22</sup> Wolf *loc cit*

<sup>23</sup> Reilly, Nolan and Lusk *Amer Journ of Physiol*, i, p. 395, 1898

Notwithstanding these complicated factors, the use of the ordinary methods of calculation sufficed to bring the average results for twenty-one hours so that the heat measured by the calorimeter was only 2.7 per cent less than that obtained by the indirect calculation.

The results would have agreed still closer, within 2 per cent, had it been possible to obtain agreements during the second and third hours after meat ingestion between the direct and the indirect method of determining the heat production. Reference to Table II will show that here the method entirely broke down. Various explanations of this phenomenon are possible. At first it seemed that perhaps in the early metabolism of protein, compounds were broken up which required more oxygen, yielded more carbonic acid and evolved less heat than the protein molecule in its entirety would have done. Also it seemed that protein itself might be oxidized in larger quantity than the nitrogen eliminated during the hour indicated. While this may in a measure be true, yet the respiratory quotients of 87 and 84 (see Table II) obtained during this second hour after meat ingestion indicate the combustion of the body's store of carbohydrates, the respiratory quotients for the non-protein part of the metabolism being respectively 99 and 91. Were the total heat production due to protein metabolized, the R. Q. would be 0.80 and if the calories of the protein metabolism exceeded the heat output of the body, and sugar from protein were retained by the organism, the R. Q. would fall below 0.80 as indeed happens in the third and subsequent hours after meat ingestion. Reilly, Nolan and Lusk<sup>24</sup> found after giving 870 grams of meat to a phlorhizinized dog weighing 35 kgms. that 2.69 grams of "extra sugar" (sugar not attributable to the nitrogen of the period) were eliminated during the first two hours after the meal. This "extra sugar" corresponds to an amount of protein metabolism represented by 0.8 gram of nitrogen. If in the dog used in the present experiment the conditions of protein metabolism were the same as in the diabetic dog, there would have been a retention of 0.8 gram of nitrogen from metabolized protein within the organism during the first two hours. If the retention were equally divided between the two hours, then the protein metab-

<sup>24</sup> Reilly, Nolan and Lusk. *loc. cit.*

olism of the second hour would be represented by an elimination of 1.29 grams N instead of 0.89 gram actually eliminated

The possibility of this picture being representative of what occurs is not denied, but there are reasons for believing that it may be an exaggerated picture. In the first place, the high respiratory quotient which is found only in the second hour does not support it. In the second place, the metabolism of ingested protein in diabetes may be much more rapid than in health. Thus, the retention of amino-acids in the organism noted by Luthje and Murlin was effected when much carbohydrate was given in the food but Ringer and Lusk<sup>25</sup> noted no such retention when the amino-acids were given in phlorhizin glycosuria.

The validity of the method of computation used, as well as the significance of the respiratory quotients as a means of interpretation, is indicated by comparing the results obtained during the second hour and during the fourteenth hour. In both hours, the carbonic acid elimination and the oxygen intake were nearly the same, whereas the nitrogen elimination in the fourteenth hour was double that of the second hour. This is shown in the following table.

	URINE N	CO <sub>2</sub>	O <sub>2</sub>	R Q	CALORIES	
					Found	Calculated
Second hour	0.89	13.13	11.03	0.87	32.86	35.97
Fourteenth hour	1.88	12.65	11.34	0.81	36.82	35.86

It is evident that recalculation of the second hour on the basis of the high protein metabolism of the fourteenth would not reveal the three calories apparently lost during the second hour.

A clearer insight into this problem resulted from suggestions made by Dr. Hans Aron during a visit to this laboratory. It had been noted that the great deficit in calories found was coincident with a considerable rise of body temperature. The question was, whether this rise in body temperature was evenly distributed throughout the animal, in which case the temperature as measured by the rectal thermometer would be a true index to the amount of heat retained in the dog, or whether there was an un-

<sup>25</sup> Ringer and Lusk *Zeitschr f physiol Chem*, lxxvi, p. 106, 1911

equal heating of different regions of the body which was not to be determined by an observation made in one locality only To decide this question, four electrical resistance thermometers were placed in the groins and axillae of the dog and the rise in skin temperature was compared with that of the rectum when the dog was lying at complete rest in the calorimeter It was found that, whereas no variation between the skin and rectal temperatures occurred during fasting, after giving meat the temperature of the skin rose far more than that of the rectum, and also, if carbohydrate were given, the skin temperature might rise while the rectal temperature remained stationary

These results are shown in Table V

It appears from this table that the increase in body temperature following the ingestion of food is not truly indicated by the rectal thermometer After giving 1200 grams of meat, the skin temperature rose  $0.42^{\circ}$  and  $0.32^{\circ}$  more than the temperature in the rectum rose, and after giving 100 grams of dextrose, the rectal temperature fell  $0.02^{\circ}$  while the skin temperature rose  $0.46^{\circ}$ , a difference of  $0.48^{\circ}$

Henriques and Hansen<sup>26</sup> cite experiments showing the temperature of a pig at different levels under the skin The size of the animal and the outside temperature unfortunately are not given The results were as follows

1 cm under the skin	33.7°
2 cm under the skin	34.8°
3 cm under the skin	37.0°
4 cm under the skin	39.0°
Rectal temperature	39.9°

Also, it has been known since the experiments of Claude Bernard<sup>27</sup> that the temperature of the liver increases after food ingestion In one instance in a fasting dog, the temperature of the hepatic vein was found to be  $38.4^{\circ}$  while in another dog after the ingestion of food, the temperature reached  $41.3^{\circ}$  in the same locality The increased heat production within the organism is accompanied by a greater distribution of warm blood to the surface of the organism

<sup>26</sup> Henriques and Hansen *Skand Archiv f Physiol*, xi, p 161, 1900

<sup>27</sup> Claude Bernard *Leçons de physiologie opératoire*, Paris, 1879

TABLE V

*Comparison of body temperatures in the dog recorded by electrical resistance thermometers in the rectum and in four places (both axillas and both groins) on the skin, the room temperature being 26° The foods given were ingested at noon*

DATE	FOOD	TIME	TEMPERATURES			
			Rectal	Increase	Surface	Increase
1911		p m				
May 17	Ordinary diet 1200 gm meat	12 45	38 14		36 61	
May 18		1 45	38 43	+0 29	36 89	+0 27
		2 45	38 44	+0 01	37 34	+0 45
				+0 30		+0 72
May 19	Fasting 1200 gm meat	12 45	37 46		35 74	
May 20		1 45	38 35	+0 89	36 86	+1 12
		2 45	38 56	+0 21	37 08	+0 22
		3 45	38 70	+0 14	37 31	+0 23
				+1 24		+1 57
May 21	Ordinary diet 100 gm dextrose	12 45	38 08		37 04	
May 22		1 45	37 99	-0 09	37 36	+0 32
		2 45	38 06	+0 07	37 49	+0 14
				-0 02		+0 46
May 23	Ordinary diet Fasting	12 45	38 26		37 02	
May 24		1 45	38 20	-0 06	37 00	-0 02
		2 45	38 02	-0 18	36 80	-0 20
				-0 24		-0 22

in order to eliminate this heat Benedict and Emmes,<sup>28</sup> however, were not able to obtain these results in man. Very likely the sweat glands maintain the skin temperature of the man at a lower relative temperature than in the dog under similar circumstances.

<sup>28</sup> Benedict and Emmes *Amer Journ of Physiol*, xxx, p 197, 1912

If one calculates the heat of metabolism retained in the body from the change in the skin temperature instead of from the change in rectal temperature, there would be an increased heat retention in the organism of 4.93 calories ( $14.7 \text{ kgms} \times 0.42^\circ \times 0.8$ ) or of 3.78 calories ( $14.7 \text{ kgms} \times 0.32^\circ \times 0.8$ ) during the second and third hours following meat ingestion. What fraction of this can be employed in recalculations in the dog obviously cannot be stated. All that can be said is that this phenomenon partly or wholly explains why the direct and indirect calorimetry almost constantly fail to agree in the dog immediately after the ingestion of food.

It is thus evident that *much of the apparent discrepancy between the "heat produced" and the "heat calculated" in the dog during the second and third hours after the ingestion of meat is due to the considerable warming of the surface of the body which is much greater than that recorded by the rectal thermometer and therefore does not enter into the computation of heat production*

Observations during the later hours failed to reveal these calories as given off to the calorimeter. This can probably be explained by the fact that the dog remained in a well-lighted, moderately cool room until the specified time for the preliminary period within the calorimeter and therefore could lose calories from his skin more readily than he could resting quietly in the chamber of the apparatus at a temperature of  $26^\circ$  to  $27^\circ$ . It is to be believed that if the animal could have remained in the calorimeter for twenty-two hours, the whole of the heat would have been recovered.

The dogs were placed in the calorimeter immediately after catheterization, the experiment began forty-five minutes or one hour after catheterization, and lasted three or four hours. Longer periods could not be attempted on account of the collection of urine and the restlessness of the dog.

#### *a The retention of protein carbon in the form of dextrose*

To the many recorded proofs of the origin of sugar from protein, first demonstrated by Claude Bernard, has finally been added that of Pflüger,<sup>29</sup> who has shown that if a fasting dog be given phlorhizin and twenty-four hours thereafter be killed, the liver contains but 0.1 per cent of glycogen, whereas if the dog in this condition be

<sup>29</sup> Pflüger and Junkersdorf *Pflüger's Archiv*, cxviii, p. 201, 1910.



given codfish in large quantity, the glycogen content of the liver may rise to 10 per cent in a few hours

Scheurer<sup>30</sup> gave between 900 and 1500 grams of meat to a dog and determined the metabolism by the Zuntz method between three and four hours after meat ingestion. He concluded from the respiratory quotient that carbon was retained from protein in the form of carbohydrate.

In the work on Dog I new confirmation of the conversion of a portion of the protein molecule into dextrose is afforded in the oxygen absorption during the various periods after the ingestion of meat. The facts are set forth in the last two columns of Table II, in Table III and especially in Table VI.

TABLE VI

*Table contrasting the actual oxygen intake with that required by theory if the carbon retention had been in the form of glycogen or of fat. Calculation according to consecutive periods run.*

TIME	CALORIES		C RETAINED (CALC AS DEXTROSE)	O <sub>2</sub> ACTUAL	O <sub>2</sub> (C RE- TAINED AS DEXTROSE)	O <sub>2</sub> (C RE- TAINED AS FAT)
	Found	Calculated				
p m						
1 45- 2 45	38 92	41 70	0 10	13 63	12 99	12 95
2 45- 3 45	40 40	41 29	1 93	13 29	12 56	11 73
3 45- 6 45	121 91	124 82	7 86	43 25*	40 61	37 23
6 45- 9 45	122 11	122 86	7 86	40 35	40 01	36 63
9 45-12 45	106 70	111 67	7 50	35 47	36 41	33 19
12 45- 1 45	35 86	36 82	3 42	11 34	12 13	10 66
1 45- 2 45	27 71	29 32	2 75	9 31	9 65	8 47
2 45- 4 45	64 24	62 36	3 08	19 56	20 19	18 87
	557 85	570 84	34 50	186 20	184 55	169 73
	Dif = 2.3 per cent			Dif = 0.9 per cent		Dif = 10 %

34.5 grams dextrose 28.3 grams N 12.1

\* Small leak in the apparatus during this period determined the day following to amount to about 1 gram O<sub>2</sub> per hour

The respiratory quotients (see Tables III and Chart I) fall during the hours of carbon retention to below that of protein itself (which is 0.80) because the unoxidized carbohydrate is retained

<sup>30</sup> Scheurer *Pflüger's Archiv*, cx, p 227, 1905

in the organism as glycogen. If the carbon were retained in the organism as fat, the respiratory quotient would rise. If one considers the period between the hours of 6.45 to 9.45 p.m., one obtains the following picture of what occurs

Calories found = 122.11, calories calculated = 122.86. C retained, calculated as dextrose = 7.86 grams. Oxygen absorbed = 40.35 grams (R.Q. = 0.77). Oxygen, calculated on the basis of the nitrogen elimination and the assumption that the protein carbon retained in the body was in the form of dextrose, = 40.01 grams (R.Q. = 0.77). Assuming that the carbon was retained in the form of fat, oxygen calculated = 36.63 grams (R.Q. = 0.85).

It is obvious from these figures that *the oxygen absorption proves the retention of carbon in the form of dextrose or glycogen in the organism*. During the fourteen hours of carbon retention following the ingestion of 1200 grams of meat, the actual oxygen absorption was 186.2 grams against a value of 184.5, calculated on the assumption that carbon was stored as glycogen or a difference of 0.9 per cent. If the carbon had been retained as fat, 169.7 grams of oxygen would have been required or 10 per cent less.

During these fourteen hours 34.5 grams of dextrose were stored as glycogen in the organism and 28.3 grams of N were eliminated in the urine. This yields a D/N ratio of 1.2:1. Since 3.6 is the maximum yield of dextrose per gram of N in diabetic urine, it is evident that one-third of the dextrose derivable from protein in metabolism was retained in the organism and deposited in the liver and other glycogen reservoirs. This represents 20 per cent of the total energy contained in the protein metabolized.

The production of dextrose from protein is not an emergency process as some writers maintain, but it is a normal function.

### *b The so-called "Specific Dynamic Action"*

The nitrogen in the urine after the ingestion of 1200 grams of meat rapidly rises to a maximum which is reached about the fourth hour and then maintains a nearly even level of elimination during a period of about ten hours, after which it slowly falls. This is shown graphically in Chart I which has been prepared from the data given in Table III.

The heat production is essentially proportional to the nitrogen elimination in the urine. The maximum specific dynamic action

appears to occur in the second and third hours. The intensity of the specific dynamic action is obtained by subtracting the heat of the protein metabolized during the period before meat ingestion from that of the hours thereafter and calculating what relation these extra calories of protein metabolism bear to the total increase in heat production for the hour

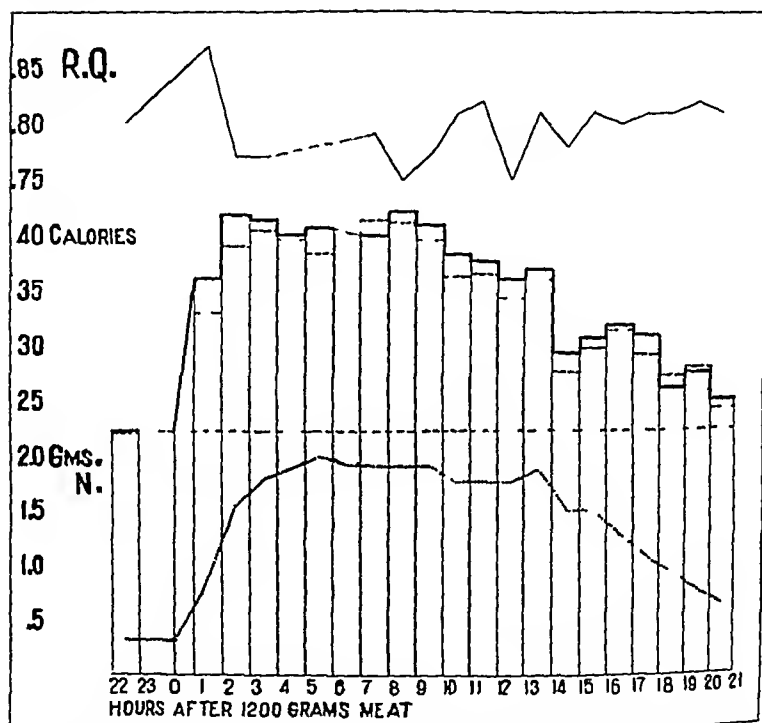


CHART 1 SHOWING THE R.Q., THE TOTAL METABOLISM DETERMINED BY INDIRECT (HEAVY BLACK LINE) AND DIRECT (BROKEN LINE) CALORIMETRY AS WELL AS THE NITROGEN ELIMINATION (DOTTED LINE) DURING HOURLY PERIODS AFTER THE INGESTION OF 1200 GRAMS OF MEAT

For the whole period the average increase in metabolism for every 100 calories of extra protein oxidized is 45 calories (If the basal metabolism be assumed to be 25 calories instead of 23.3 then the specific dynamic action of 100 calories of protein is represented by 38 additional calories of heat production)

If the figures obtained were calculated after the fashion of Rubner, the results for the whole period would be similar to those obtained by him. That is, the ingestion of meat containing 900 calories in protein caused an increase in metabolism of 270 calories during a period of twenty hours, or the ingestion of 100 calories caused an increase of 30 calories in heat production.

The specific dynamic action of protein during the second and third hours after meat ingestion is greater than it is during the other periods. During the second hour it is represented by 90, and during the third hour by 60. That is to say, the heat production rises almost to its maximum during the second hour even though the increase in protein metabolism as indicated by the nitrogen elimination has reached only a third its height. Various explanations occur. It might be due to 'Darmarbeit' in the sense of Zuntz, but this is improbable because the same high metabolism is maintained in the fourteenth hour after taking food when three-quarters of the nitrogen of the ingested protein has been eliminated in the urine and the work of the intestinal canal must be largely completed. It might be due to the fact already discussed of a lag in the nitrogen excretion, which is contradicted by the respiratory quotients obtained.

That the factor of "Darmarbeit" is not important is shown by the work of Benedict and Emmes<sup>11</sup> who have demonstrated that cathartics and agar agar given to man have no effect on metabolism.

There are three conceivable explanations of the increased heat production caused by meat ingestion.

- 1 The large quantity of varying amino-acids entering the organism furnish multifarious compounds readily oxidizable by the cells in the sense of Voit.

- 2 They furnish compounds, some of which are used for cell dynamics, while others yield free heat to the body in the sense of Rubner.

- 3 They are at first retained unoxidized in the organism as shown by Luthje, Murlin, Wolf and Folm, and in combination or in contact with the protoplasm of the cells act as stimuli to increased oxidation.

On this last hypothesis, after giving meat, the resulting inrush of amino-acids would bring about a direct stimulus to the cells, causing an increased heat production. This would explain the

<sup>11</sup> Benedict and Emmes *Amer Journ of Physiol*, 111, p 197, 1912

greatly augmented metabolism of the second hour after food ingestion in the absence of any indication of a correspondingly increased protein metabolism

*B The results of the ingestion of 700 grams of meat in Dog I*

The details of these experiments are presented in Table VII and the essential points are given in Table VIII. The urinary analyses are to be found in the Appendix, Table XI.

The direct and indirect calorimetry agree within 0.7 per cent during a period of seven hours.

During the second hour the discrepancy between the heat produced and the heat calculated is not always as great as after giving 1200 grams of meat. The specific dynamic action of the meat reaches 139, 106 and 69 in the second, third and fourth hours, respectively. With a non-protein respiratory quotient of 0.90 and a specific dynamic action equal to 139 in the second hour after giving 700 grams of meat, it seems certain that the great rise in metabolism which takes place before there is any very great nitrogen elimination in the urine, is really due to a direct stimulation of the cells by the entering amino-acids.

It appears that, whereas 1200 grams of meat causes an increase in metabolism from 22 calories per hour to 40 calories, an increase of 18 calories or 82 per cent, 700 grams of meat causes an increase from 22 to 32 calories, an increase of 10 or 45 per cent. Since these figures are nearly proportional (700/1200 = 10/17) it follows that the increase in heat production after giving these quantities of meat is proportional to the quantity of meat ingested.

That the intensity of the heat production (excluding the first few hours) is very nearly proportional to the nitrogen elimination in the urine appears from the following comparison.

1200 GRAMS MEAT			700 GRAMS MEAT		
Hours After Food	N in Urine	Calories	Hours After Food	N in Urine	Calories
15	1.50	29.32	6	1.55	32.51
16	1.50	30.65	7	1.54	32.07

In the one case, at the end of the sixteenth hour, 10 grams out of 36 grams of nitrogen ingested had not been eliminated in the urine, in the other case, 13.8 grams out of 21 ingested had not been eliminated after seven hours.

BODY TEMPERATURE			MORNING WEIGHT	C TO BODY	DEXTROSE TO BODY	O <sub>2</sub> CALC	O <sub>2</sub> FOUND
Start	End	Dif					
			<i>kg</i>	<i>gram</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
38 28	38 53	+0 21	14 24				
38 86	39 20	+0 34	14 03				
37 62	37 90	+0 28	16 07				
37 37	37 89	+0 28					
38 28	38 48	+0 20	13 80				
38 47	38 59	+0 12	13 92				
38 46	38 52	+0 06	14 24				
38 48	38 58	+0 10	13 4				
38 58	38 72	-0 14	13 4	0 85	2 13	10 51	11 80
38 54	38 64	+0 10	13 5	0 91	2 28	10 61	10 86
38 64	38 72	+0 08	13 5	0 91	2 28	10 61	11 70
38 72	38 70	-0 02	13 5	0 01	0 02	11 51	12 11

7) at noon

N AFTER 12 45	EXTRA CALORIES OFF PROTEIN METAB	INCREASE IN METAB ABOVE FASTING IN CALORIES	EVERY 100 CALORIES OF PROTEIN IN- CREASES METAB IN CAL
<i>gram</i>			
0 52	6 94	9 55	139
1 33	13 09	13 83	106
2 30	16 89	11 62	69
3 82	31 87	9 94	31
5 37	32 73	10 25	31
6 91	32 29	9 79	33
7 15	24 60	10 65	43
	158 41	75 63	48

34

*The intensity of the metabolism must be due either to the intensity of the intermediary processes of protein metabolism itself or, more probably, to the degree of mass action of amino-acids acting as stimuli upon the cellular protoplasm*<sup>32</sup>

*C The results of the ingestion of 1000 grams of meat in Dog II*

Table IX presents the results of the ingestion of meat compared with the early morning minimum. When 1000 grams of meat were given, 500 grams having been ingested the day before, the metabolism rose at once from the basal level of 16.2 to 29.6 calories, an increase of 13.4 calories or 83 per cent. This increase of 83 per cent exactly corresponds to that in Dog I after giving 1200 grams of meat. However, the nitrogen in the urine was not eliminated as freely as in Dog I. That the upper limit of absorption in Dog II was reached after administering 1000 grams of meat was evidenced by occasional partial vomiting of the food, which was, however, afterwards eaten. The dog was not absolutely quiet in this experiment, making a single movement (perhaps raising the head?) every two to four minutes. Attention need only be called to the usual discrepancy between calories found and calories calculated during the second and third hours following food ingestion.

The character of the hourly nitrogen metabolism in this dog after giving meat has already been indicated in Table IV and to that the following data may be added

TABLE X

DATE	TIME	TOTAL N	TOTAL N PER HOUR	FOOD
1912		grams	grams	
May 1	12 00-4 08	3 348	0 810	1000 grams meat at noon
	5 00-6 00	1 235	1 235	
May 2				500 grams meat at noon
May 3	12 00-1 00		0 376	1000 grams meat at noon
	1 00-2 00		0 716	
	2 00-3 00		0 895	
	3 00-7 08	5 040	1 218	

<sup>3</sup> This paper was completed before the publication of Folin and Denis (this *Journal*, vii, p. 141, 1912) in which was stated, "If such is the case, each tissue maintains a certain supply of each amino-acid, and the urea formation from any particular amino-acid, depends, so to speak, on the 'partial pressure' of that particular acid."

## IV SUMMARY

1 A dog weighing 13.5 kgms, the heat production of which was 22.3 calories during an hour previous to food ingestion, was given 1200 grams of meat at noon. The heat production rose to 36 calories in the second hour and 42 in the third. It was maintained above 40 calories per hour through the tenth hour. In the fourteenth hour, it had fallen to 37 calories and then remained at 30 calories up to the eighteenth hour, falling rapidly to 25 calories in the twenty-first hour.

2 Ingestion of 700 grams of meat by the same dog caused an increase in metabolism which was less than that caused by 1200 grams, but in the two cases the increase was proportionate to the quantity ingested.

3 The increased metabolism was proportional to the nitrogen elimination, except in the second and third hours. In the second hour the metabolism rose almost to its maximum although the urinary nitrogen reached only a third of its maximum. Since the non-protein respiratory quotient for this period was often above 90, it appears that carbohydrate and not additional protein was oxidized during this hour. On this is based the argument that the incoming amino-acids, in proportion to their mass action, stimulate the protoplasm to higher oxidation.

4 After giving 1200 grams of meat the hourly heat production is directly measured by the calorimeter essentially corresponded to that as calculated indirectly from the urinary nitrogen, the carbonic acid output and oxygen intake, except in the cases of the second and third hours.

5 The reason that the calculated heat production does not always agree with that actually found during the second and third hours is largely due to the fact that the rectal temperature of the dog does not give a measure of the temperature increase of the whole dog. This was shown by the fact that the skin temperature rises to a greater extent than the rectal temperature after the ingestion of food.

6 During fourteen hours after giving 1200 grams of meat, carbon derived from protein metabolism was retained in the organism. This carbon was retained as dextrose because calculations based on this assumption showed that the actual quantity of oxygen ab-



sorbed agreed within 0.9 per cent of that required, whereas, if carbon had been retained in the form of fat, 10 per cent less oxygen would have been required. The dextrose retained in relation to nitrogen eliminated manifested the relation,  $D/N = 1.21$ .

7 After giving 1200 grams of meat it may be calculated for the whole period that for every 100 calories of protein oxidized above that oxidized during the morning hour before food ingestion, there may be an additional heat production in the animal amounting to 45 calories.

8 Intestinal work (Darmarbeit) appears to have little to do with the increased heat production because a high metabolism is maintained in the fourteenth hour after food ingestion even though three-quarters of the nitrogen of the protein administered has been eliminated in the urine and the work of the intestinal canal must have been largely completed.

#### APPENDIX

Showing urinary analyses "Residual" N is the estimated quantity for a fraction of an hour.

roman 9 00 a m to 7 45 p m

DEC 23 EXP 9	FEB 21 EXP 19	MAR 13	MAR 17 EXP 20	APR 21	APR 24 EXP 23	APR 28 EXP 31
0 300	0 345					
0 132	0 397	0 397	0 397	0 465	0 400	0 465
0 756 0 546	0 892	0 892	0 892	0 942	0 840	0 942
1 434 1 529 at 2 25 p m						
	1 273	1 273	1 273	1 283	1 180 0 295	1 283 0 257
					2 716 2 776 at 3 00 p m	2 947 2 656 at 2 55 p m
	1 402 0 367	1 402	1 402			
	4 331 4 329 at 4 00 p m					



all values in roman 9 00 a m to 8 45 p m

9	MAR 14	MAR 18	MAR 31	APR 11	APR 13	APR 15	APR 17	MAY 1
4		0 344			0 463	0 472	0 318	
	0 178	0 38	0 28	0 28	0 28		0 28	0 178
	0 886	0 87	0 89	0 89	0 89	1 20	0 89	0 886
4	1 327	1 74	1 55	1 55	1 55	1 81	1 34 2 51 2 413 at 2 37 p m	1 327
5	1 625	1 80	0 77	1 80	0 45			0 27
m			3 49 3 397 at 3 09 p m	4 52 5 27	3 17 3 07 at 3 00 p m			2 66 2 567 at 2 55 p m + 27 23 to noon = 29 80 in 24 hrs
		0 45 5 24 5 075 at 4 00 p m		2 024				
				2 024	1 44			
				1 871	1 92			
				1 838	1 92			





After considerable search, an enzyme was selected which permitted the isolation of one of the nucleosides entering into the structure of the molecule of the complex nucleic acid of animal origin. The substance obtained is guaninehexoside. It was semicrystalline, soluble in hot alcohol and separated from this solution on cooling. The substance has the composition  $C_{11}H_{15}N_5O_6$ . It did not reduce Fehling's solution and with silver nitrate formed a precipitate only when the solution was neutral. The silver compound immediately dissolved in a minimal excess of ammonia water. It did not give the orcin test on direct boiling with hydrochloric acid but gave the test with hydrochloric acid containing some copper. After hydrolysis the substance, on boiling with Fehling's solution, formed a voluminous white precipitate of cuprous guanine compound. From the products of hydrolysis were obtained an osazone melting at  $198^{\circ}C$  and guanine sulphate.

#### EXPERIMENTAL PART

The reaction mixture of nucleic acid and enzyme was made alkaline with ammonia and to the mixture 98 per cent alcohol was added as long as a precipitate formed. This was removed by filtration, and the mother liquor concentrated to dryness under diminished pressure. This was again dissolved in water, the solution rendered alkaline with an excess of ammonia and the treatment with alcohol repeated. The final filtrate was again concentrated to dryness under diminished pressure. The residue was taken up in water and the nucleotides still present in the solution removed by means of a solution of basic lead acetate. The precipitate thus formed was removed by filtration and to the filtrate ammonia and more lead acetate solution added as long as a precipitate formed. The last precipitate was filtered and washed carefully with cold water. It was finally suspended in water and decomposed by means of hydrogen sulphide. The filtrate from lead sulphide was rendered slightly alkaline by means of ammonia and evaporated under diminished pressure to a thick syrup. (It is very important not to omit this step, since the purine hexosides are easily decomposed even by acetic acid.) On cooling, the syrup is transformed into a gelatinous mass resembling crude guanosine. The substance was then filtered on a suction flask over silk and

then dissolved in hot alcohol, filtered and allowed to cool. A sediment was formed on cooling, composed of rosettes resembling impure leucine.

The analysis of this substance gave the following data

0.1120 gram of the substance gave 0.1746 gram of  $\text{CO}_2$  and 0.0538 gram of  $\text{H}_2\text{O}$ .

0.1500 gram of the substance employed for a Kjeldahl nitrogen estimation required for neutralization 23.5 cc. of  $\frac{N}{10}$  sulphuric acid.

	Calculated for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_2$	Found
C	42.20	42.50
H	4.84	5.34
N	22.30	21.93

For hydrolysis about 0.200 gram of the substance was dissolved in 2 per cent sulphuric acid and heated on a water bath with return condenser for half an hour. The base was removed with silver oxide. Silver purine was filtered and decomposed with hydrochloric acid. The filtrate from the silver chloride was allowed to stand in the refrigerator and a sediment of guanine hydrochloride formed. The hydrochloride was transformed into the free base by means of ammonia and this again transformed into the sulphate.

The filtrate from the silver purine was freed from silver and the free sulphuric acid neutralized with sodium acetate. The clear solution served for the preparation of the phenylosazone. For purification it was recrystallized out of water containing very little pyridine. The osazone had the melting point of  $198^\circ\text{C}$ .





## ON CEREBRONIC ACID

By P A LEVENE AND W A JACOBS

(From the Rockefeller Institute for Medical Research, New York)

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Cerebronic acid was first discovered by Thudichum,<sup>1</sup> who erroneously considered it an isomer of stearic acid and named it neurostearic acid. It was the merit of Thierfelder to correct the view of the first discoverer and to demonstrate that the substance was an oxyacid of the composition  $C_{25}H_{50}O_3$  and not  $C_{18}H_{36}O_2$ . In justice to Thudichum it must be stated that his analytical data agreed sufficiently with the figures required by the new formula for the substance. There is, however, a considerable disagreement in the data of the two observers in regard to the melting point of their substances, Thudichum recording the melting point at 84–85°C and Thierfelder<sup>2</sup> at 98–99°. On the basis of this there was still open the possibility that the two substances had a different structure. Hence it was urgent to establish the relationship of the two substances.

Concerning the structure of cerebronic acid, it was established by Thierfelder that it contained one hydroxyl group, and it was also made very probable by him that the molecule contained twenty-five carbon atoms. It is well known that the percentage composition of carbon and hydrogen in the higher fatty acids does not furnish sufficient information regarding the number of carbon atoms in the molecule. With a greater degree of probability this can be established on the basis of the "acid value" of the substance.

Regarding the place of the hydroxyl group and regarding the character of the linking between the carbon atoms the work of

<sup>1</sup> Thudichum *Die chemische Konstitution des Gehirns*, Tübingen, 1901, pp 194, 195

<sup>2</sup> *Zeitschr f physiol Chem*, xliii, p 21, 1904

Thierfelder offered no information. The work of this author also failed to take cognizance of the optical activity of the substance.

The results of the present investigation led to the conclusion that cerebronic acid is the normal  $\alpha$ -hydroxypentacosamic acid. In the hydrolysis mixture it occurs in the form of two isomers, the optically active dextrorotatory form of  $[\alpha]_D^{30} = +4.16^\circ$  and the optically inactive form. The active substance melts at a point somewhat higher than given by Thierfelder,<sup>3</sup> being 106–108°C, the inactive at 82–85°C. Thus, apparently, Thudichum had in his hand the optically inactive substance, while Thierfelder's was undoubtedly a mixture of the two. It is possible, of course, that a substance with still higher rotatory power may be obtained.

The place of the hydroxyl group was established by the fact that on oxidation with alkaline permanganate solution the cerebronic acid gave rise to an acid of the composition  $C_{24}H_{48}O_2$ .

The normal character of the carbon chain was made probable by the transformation of cerebronic acid into a hydrocarbon that melted between 54–57°C. According to Kraft<sup>4</sup> and Marie<sup>5</sup> the three nearest hydrocarbons have the following melting points:  $C_{24}H_{50}$ , 51°C,  $C_{25}H_{52}$ , 53.5°–54.0°C,  $C_{26}H_{54}$ , 58°C.

Thus it seems very probable that the hydrocarbon obtained from cerebronic acid had the composition  $C_{25}H_{52}$ . This assumption is further substantiated by the fact that the acid value for the pure cerebronic acid corresponded to molecular weight value of  $C_{25}H_{50}O_3$ .

The optically active substance was separated from the inactive by fractional precipitation with lithium acetate.

## EXPERIMENTAL PART

### *Preparation of cerebronic acid*

The acid was obtained from cerebrine. Fifty grams of the cerebroside were taken up in 1500 cc of methyl alcohol containing 70 grams of sulphuric acid to each 1000 cc. The mixture was

<sup>3</sup> *Zeitschr f physiol Chem*, xlii, p 21, 1904

<sup>4</sup> *Ber d deutsch chem Gesellsch*, xv, p 1711, 1882

<sup>5</sup> *Bull de la soc chim*, xv, p 567, 1893

heated with return condenser in a water bath for six hours and the separated acid and ester allowed to cool at  $0^{\circ}\text{C}$ . They were then filtered on suction over silk and boiled with an excess of alcoholic soda in a water bath with return condenser for about four hours. The mixture was then again allowed to cool and the soaps filtered off. These were dissolved in glacial acetic acid containing enough hydrochloric acid to combine with the base of the soap. The solution was then poured into a large volume of distilled water from which the free acid separated. It was then filtered and dissolved in boiling alcohol, from which it separated on cooling.

#### *Purification of the crude cerebronic acid*

Several processes were used for this purpose, but none of them can as yet be recommended as final. Considerable effort was lost in an attempt to obtain an acid with a very sharp melting point. It was found that the acid melting at a higher temperature was less soluble in ether, petroleic ether (boiling at  $60^{\circ}\text{C}$ ) and ligroin (boiling at  $80^{\circ}\text{C}$ ). The purification was therefore accomplished by repeated recrystallizations of the substance from one of the three solvents as long as the melting point was rising. The final substance obtained in that manner had a melting point of  $102^{\circ}\text{C}$ , which, however, was not very sharp. This substance was further purified by fractionating it out of a methyl alcoholic solution with an alcoholic solution of lithium acetate. In this instance the acid obtained from the top fraction had a fairly sharp melting point at  $106\text{--}108^{\circ}\text{C}$ . The substance obtained from the lower fraction had a melting point of  $82\text{--}84^{\circ}\text{C}$ .

#### *Ultimate analysis of the two acids*

Not less than twenty different samples of different melting points were analyzed. They all agreed well with the theory and here will be given only three of them, the first melting at  $106\text{--}108^{\circ}\text{C}$ , the second at  $95^{\circ}\text{C}$  and the third at  $82\text{--}84^{\circ}\text{C}$ .

- (1) 0.1224 gram of the substance gave 0.3388 gram  $\text{CO}_2$  and 0.1360 gram  $\text{H}_2\text{O}$
- (2) 0.1206 gram of the substance gave 0.3342 gram  $\text{CO}_2$  and 0.1356 gram  $\text{H}_2\text{O}$
- (3) 0.1194 gram of the substance gave 0.3292 gram  $\text{CO}$  and 0.1350 gram  $\text{H}_2\text{O}$

	Calculated for $\text{C}_{21}\text{H}_{30}\text{O}_2$	1	Found 2	3
C	75.33	75.42	75.40	75.60
H	12.50	12.43	12.65	12.57

*Optical activity of the acids*

Since the acids had the same percentage composition and differed in their melting points it was natural to look for the cause of it in possible stereoisomerism, since the acid contains a hydroxyl group in the molecule. As was stated already, the substance with the highest melting point had also the highest optical activity.

0.2190 gram of cerebronic acid (M.P. = 106°C) dissolved in 5.0 cc of pyridine. Total weight = 5.0126 grams, showed a rotation of +0.35° in pure yellow light and in a 2 dm tube at 20°C

$$[\alpha]_D^{20} = +4.01^\circ (\pm 0.00^\circ)$$

0.2229 gram of the same substance fractionated once more by means of lithium acetate was dissolved in 5 cc of pyridine. Total weight = 5.0960 grams. Rotated in 2 dm tube and in pure yellow light 0.37° to the right

$$[\alpha]_D^{20} = +4.16^\circ (\pm 0.01^\circ)$$

0.2667 gram of cerebronic acid melting at 92°C was dissolved in 5 cc of pyridine. Total weight = 5.0763 grams. Rotation in 2 dm tube in pure yellow light = +0.10°

$$[\alpha]_D^{20} = +0.965^\circ (\pm 0.00^\circ)$$

0.2000 gram of cerebronic acid, melting at 82-84°C, dissolved in pyridine. Total weight = 5.0578 grams. Proved inactive

*Molecular weight estimation*

The molecular weight was determined by titration with an  $\frac{N}{2}$  solution of sodium hydrate. The acid was dissolved in a mixture of benzol and pure methyl alcohol. It is not always an easy matter to obtain the acid of sufficient purity for the purpose. The easiest way of obtaining the substance is by way of the lead salt.

(1) 1.4394 grams of the acid dissolved in benzol + methyl alcohol. Titrated with  $\frac{N}{2}$  alkali. Phenolphthalein used as indicator. Result calculated to  $\frac{N}{10}$  value = 36.00 cc

(2) 1.5896 grams of cerebronic acid treated in the same manner as the previous required 47.25 cc of  $\frac{N}{10}$  alkali

(3) 1.3073 grams cerebronic acid treated as above required 32.2 cc of  $\frac{N}{10}$  alkali

(4) 2.2440 grams cerebronic acid treated as above required 57.5 cc of  $\frac{N}{10}$  alkali

(5) 1 5000 grams cerebronic acid required for titration 37 8 cc of  $\frac{N}{10}$  alkali

M W	Calculated for	1	2	Found	4	5
	$C_{24}H_{48}O_2$			3		
	398 0	399 0	397 0	406 0	396 8	397 0

*Determination of the place of the hydroxyl group in the cerebronic acid*

This was determined by oxidation of cerebronic acid by an alkaline solution of potassium permanganate <sup>6</sup> Numerous experiments were performed, the conditions of oxidation remaining unchanged They were the following

Nine grams of analytically pure cerebronic acid were suspended in 300 cc of water containing 3 grams of potassium hydrate and heated until the acid formed a gelatinous soap This was then added to a hot solution of potassium permanganate, made up of 1 liter of water and 4 5 grams of potassium permanganate The heating was continued until the color of permanganate changed to a brownish tint The boiling was then discontinued and the mixture decolorized by means of sodium bisulphite The insoluble soaps generally collected on the surface of the fluid

The subsequent treatment differed in individual experiments In some the soaps were immediately transformed into the free acids, and this fractionated by means of ether and of ligroin In other experiments it was attempted to first separate the salts on the basis of the differences of their solubility in alcohol

The most convenient procedure is the following Separate the sodium salts into two fractions, one soluble in hot alcohol, the other insoluble The latter fraction consists principally of unchanged cerebronic acid The hot alcoholic solution of soaps is allowed to cool until the soaps separate out These are filtered and converted into the free acids In this fraction the acid  $C_{24}H_{48}O_2$  predominates Its purification is based on the property of its lithium salt to remain insoluble in boiling methyl alcohol The lithium salt is converted into the free acid For final purification it was transformed into the lead salt and this again reconverted into the free acid

<sup>6</sup> Edmed *Journ Chem Soc*, lxxiii, pp 627-634, 1898

*Analysis of the acid  $C_{21}H_{40}O_2$* 

- (1) 0 1220 gram of the acid gave 0 3492 gram  $CO_2$  and 0 1382 gram  $H_2O$   
 (2) 0 1238 gram of another sample gave 0 3568 gram  $CO_2$  and 0 1400 gram  $H_2O$   
 0 1228 gram of the second sample gave 0 3550 gram  $CO_2$  and 0 1428 gram  $H_2O$   
 (3) 0 1214 gram of a third sample gave 0 3504 gram  $CO_2$  and 0 1400 gram  $H_2O$   
 0 1212 gram of the third sample gave 0 3480 gram  $CO_2$  and 0 1400 gram  $H_2O$   
 (4) 0 1212 gram of the substance gave 0 3470 gram  $CO_2$  and 0 1446 gram  $H_2O$

	Calculated for $C_{21}H_{40}O$	1	2	Found	3	4
C	78 20	78 10	(a)78 45 (b)78 75	(a)78 70 (b)78 40		78 40
H	13 16	12 69	(a)12 72 (b)12 99	(a)12 91 (b)12 92		13 40

*Analysis of the sodium salt of the new acid*

0 1636 gram of the sodium salt gave 0 0300 gram  $Na_2SO_4$ ,  $Na = 5.94$  per cent

	Calculated for $C_{21}H_{38}O_2Na$	Found
Na	5 90	5 94

*Molecular weight estimation of the new acid*

0 9500 gram of the acid dissolved in benzol and methyl alcohol and titrated with  $\frac{N}{10}$  alkali, figures given in  $\frac{N}{10}$  alkali Required, 26.5 cc of  $\frac{N}{10}$  alkali

0 9578 gram of the same substance required for neutralization 25.75 cc of  $\frac{N}{10}$  alkali

	Calculated for $C_{21}H_{40}O_2$	Found	II
M W	368 0	375 0	371 0

*Melting point of the new acid*

The melting point of the new acid was at 81–82°C. Of the two known acids having the composition  $C_{21}H_{40}O_2$  one, lignoceric, melts at 80.5°C and the other, carnaubic, melts at 72.5°. It is possible that the substance is identical with lignoceric acid.

*Reduction of cerebronic acid with hydronodic acid*

The object of the first experiment under the conditions to be here described was to reduce the hydroxyl group and thus to obtain an acid of the composition  $C_{25}H_{50}O_2$ . Unexpectedly a sub-

stance was obtained containing considerable phosphorus, but the organic radicle of the substance possessed the composition of a hydrocarbon. The experiments were all repeated under identical conditions. It is possible that the yield might have been improved by following the directions of Kraft<sup>7</sup> for the reduction of fatty acids to the corresponding hydrocarbons, but the simplicity of this process was very attractive.

Lots of 2 grams of the acid with 10 cc of hydroiodic acid, of specific gravity 1.96, and 0.5 gram of red phosphorus were heated in sealed tubes at 125°C for four hours. The tubes were then opened, the hydroiodic acid diluted with an equal volume of water, again sealed and heated at 110–115°C for about three hours. The product of reaction had a white solid appearance. It was washed with water, dissolved in hot alcohol and allowed to cool. The substance solidified and was filtered. The substance was again dissolved in alcohol, a few drops of phenolphthalein added and rendered alkaline by means of an alcoholic solution of sodium hydrate. The solution was evaporated to dryness. The dry mass was transferred to a flask and distilled at about 0.5 mm pressure. The distillate was purified by redissolving in hot alcohol and allowing the solution to cool. The substance was then filtered and freed from adhering alcohol by melting.

Many samples were analyzed. The analysis of two will be reported here.

0.1300 gram of the substance gave 0.4092 gram of CO<sub>2</sub> and 0.1673 gram of H<sub>2</sub>O.

0.1041 gram of the substance gave 0.3277 gram of CO<sub>2</sub> and 0.1337 gram of H<sub>2</sub>O.

	Calculated for C <sub>23</sub> H <sub>42</sub>	Found	
		I	II
C	85.10	85.30	85.70
H	14.90	14.31	14.37

### *The melting point of the hydrocarbon*

The melting point of one sample of the hydrocarbon was 53–56°C, of another, 54–57°C. Kraft determined the melting point of the normal tetracosan at 51.1°, of heptacosan 59.5°C, and Marie found for pentacosan 53.5–54°C. Perhaps if we had suffi-

<sup>7</sup> *Ber d deutsch chem Gesellsch*, xv, p 1687, 1882



cient material for a second distillation of the paraffin the melting point might have come down to that of Marie. On the other hand, the difference in the melting points of normal, tri- and tetracosan is  $3.4^{\circ}\text{C}$ , and according to this the melting point for pentacosan may be expected to be  $54.5^{\circ}\text{C}$ , against  $53\text{--}56^{\circ}\text{C}$  found for our substance.

# ON THE CEREBROSIDES OF THE BRAIN TISSUE

By P A LEVENE AND W A JACOBS

(From the Rockefeller Institute for Medical Research, New York )

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Under the general name of cerebrosides have been described a number of individual substances designated by special names and apparently possessing distinct chemical and physical properties. The exact relationship of the substances one to another has not been fully established. The subject has been treated in a critical way in several exhaustive articles written by men as competent as Frankel,<sup>1</sup> Leathes,<sup>2</sup> Bang<sup>3</sup> and Cramer<sup>4</sup>. One is surprised to find that the views of these writers are in disagreement on some of the fundamental points regarding the relationship of different cerebrosides. The better known substances of this group are phrenosine and kerasine of Thudichum,<sup>5</sup> cerebrine, homocerebrine and encephaline. The first two of the latter three were studied by Parcus<sup>6</sup> and by Kossel and Freytag<sup>7</sup>. Finally to the same group belong cerebrin of Thierfelder and pseudocerebrine of Gamgee. Bang, the most skeptical of the writers, believes in the existence of only two cerebrosides—phrenosine and kerasine. Cramer and Leathes are inclined to distinguish between cerebrine and cerebrin, and admit the individuality of kerasine and of homocerebrine. The latter two substances they consider identical. Also Frankel is inclined to regard cerebrine, cerebrin, pseudocerebrine and phren-

<sup>1</sup> *Ergeb d Physiol*, viii, p 212, 1909

<sup>2</sup> *The Fats*, Longman, Green and Co, 1910

<sup>3</sup> *Chemie und Biochemie der Lipide*, Wiesbaden, 1911

<sup>4</sup> *Biochemisches Handlexicon*, iii, p 225, 1911

<sup>5</sup> *Die chemische Konstitution des Gehirns*, 1901

<sup>6</sup> *Journ f prakt Chem*, xiv, p 310, 1881

<sup>7</sup> *Zeitschr f physiol Chem*, vii, p 431, 1893

osine as one substance. On the basis of personal experience this view was also defended by Gies and Posner<sup>8</sup>

However, it must be remarked that the views of most of the writers are not supported by indisputable experimental proof. The hypotheses are mostly a matter of personal preference, and most writers express them with great caution. The difficulty in coordinating the data on the different cerebrosides is largely due to the fact that the investigations have been carried out by different workers at very distant intervals and no one worker attempted to coordinate his results with those of his predecessors. It was realized that three principal cerebrosides must be closely related one to another, but it was not clear wherein the point of distinction was contained.

It was the aim of the investigation, here to be presented, to search for these.

As there existed complete accord regarding the identity of the base and of the carbohydrate entering into the composition of phrenosine, cerebrine, cerebrin and kerasine, their point of distinction has to be looked for in the fatty acids that helped to make up their molecules. In reality the data regarding the melting points and the percentage composition of the fatty acids present striking differences. Hence our attention was directed to the study of the fatty acids of the mixed cerebrosides as obtained by the process of Parcus. At first it seemed as though an acid could be isolated that was richer in carbon than cerebronic acid and which had a very low melting point. But on purification of that substance it always showed the composition of cerebronic acid, although it always possessed a low melting point. It was then discovered that the acid with the low melting point was the optically inactive cerebronic acid. Thus no evidence was obtained in favor of the view that another acid than cerebronic acid was present among the decomposition products of the mixed cerebrosides. If it was present its quantity undoubtedly was very small. Hence, the cerebrosides referred to in this publication seem to be identical from the standpoint of the character of their components.

Another fact supporting the view of the chemical identity of the cerebrosides was found in the following

<sup>8</sup> This *Journal*, 1, p. 59, 1905

The mixed cerebrosides were fractionated first according to Parcus on the basis of the difference in their solubility in alcohol. Each fraction was then recrystallized from glacial acetic acid. Under this condition from cerebrine and from kerasine substances were obtained that possessed the same composition and the same melting point as cerebrin and hence, could be both regarded as cerebrine or cerebrin. In the glacial acetic acid mother liquor there remained a small quantity of substance that was extremely soluble in glacial acetic acid and in alcohol at room temperature. The proportion of this substance was very small and as yet has not been further studied. However, the fact was made clear that the major part of cerebrine and of kerasine was composed of the substance having the composition of cerebrin. And yet, when entirely free from impurities, the different fractions differed in their solubility in alcohol and in their physical appearance as they settled out of alcohol. The differences were the same as described by Parcus for cerebrine and homocerebrine.

The similarity of cerebrine, cerebrin, and kerasine is also supported by the bromine estimation made by Kossel and Freytag. There is only one double bond in the molecule of cerebrin, which could lead to the formation of a dibrom derivative, and not a tribrom body as assumed by Kossel and Freytag. The theory for a dibrom cerebrin requires 16.22 per cent of bromine, whereas Kossel and Freytag found 16.60 and 16.30 per cent for cerebrine and 16.90 and 17.25 per cent for kerasine.

It is also noteworthy that in all the three cerebrosides the primary amino group of the sphingosine is substituted, as none of them, treated with nitrous acid in the apparatus of Van Slyke, gives rise to nitrogen gas.

A difference appeared only when the optical activity of the substance was studied. It was found generally that the substances obtained from the cerebrine fraction were dextrorotatory, while those obtained from the kerasine fraction were generally optically inactive, and once a sample was obtained from the kerasine fraction that was strongly levorotatory. Furthermore, by means of a mixture of pyridine and alcohol it was found possible to fractionate the dextrorotatory substance into a fraction practically inactive and into another with a dextrorotation higher than that of the original substance.

Since the three cerebrosides are distinguished principally by differences in solubility and since there is no evidence to the effect that they are composed of different elements, one may feel justified in advancing the hypothesis that the three cerebrosides are all mixtures of stereoisomeric substances. The difference in the solubility of the various substances may be determined by the proportion of the optically active and inactive substances. It would perhaps be simpler to abandon most of the nomenclature and to refer to cerebrosides as *d*-cerebrine, *l*-cerebrine and *dl*-cerebrine. Of course, it still is necessary to discover a method by which a separation could be accomplished conveniently. Cerebrine, cerebrin and phrenosine correspond to *d*-cerebrine, kerasine and homocerebrine to *dl*-cerebrine. Of course the possibility is not excluded that the lipoids of the brain contain cerebrosides of an entirely different order.

#### EXPERIMENTAL PART

##### *Preparation of cerebrosides*

Part of the cerebrosides were prepared following the directions of Parcus, the other part by a slight modification of the process. The details were the following. Brains freed from the membranes were passed through a hashing machine and suspended in a barium hydrate solution. The mixture was brought to a boil and the solid brain tissue separated from the liquid by filtering through cheese cloth. The residue was then suspended in 95 per cent alcohol and the mixture was brought to a boil in a percolator. The alcohol was then removed and the extraction continued with 95 per cent alcohol containing 5 per cent of ammonia water. The extraction was continued as long as the alcohol gave a precipitate on cooling. The combined alcoholic extracts were placed in a refrigerator at  $-1^{\circ}\text{C}$ . A sediment formed on standing. This was again dissolved in 95 per cent alcohol and again placed in the refrigerator. The precipitate of mixed cerebrosides thus formed was then extracted by means of absolute alcohol in a water bath at  $65^{\circ}\text{C}$ . The alcoholic filtrate was placed in a thermostat and there allowed to cool. The precipitate thus formed was considered as cerebrine in the sense of Parcus. In the early phases of the work the mother liquors from cerebrine were allowed to stand at room

temperature over night, which gave rise to an intermediate fraction, the mother liquor from this was placed in the refrigerator, and the precipitate there formed was regarded as homocerebrine or kera-sine. Later the intermediate stage was omitted and the mother liquor from cerebrine placed immediately in the refrigerator. The traces of ash still present were removed according to the directions of Parcus.

*Proportion of the three fractions in the mixed cerebroside*

About 35 grams of the mixed cerebroside dissolved in absolute alcohol were allowed to stand in the thermostat room over night the precipitate, thus formed, filtered and dried in a vacuum desiccator, weighed 16.5 grams or 47.4 per cent. From the mother liquor of this at room temperature separated out 4 grams or 11.4 per cent of the second fraction, and finally, from the second mother liquor separated in the refrigerator at  $-1^{\circ}$ , 14.2 grams of the third precipitate or 40.6 per cent of the total cerebroside.

Precipitate I = 47.4 per cent of total

Precipitate II = 11.4 per cent of total

Precipitate III = 40.6 per cent of total

*Purification of the fractions*

*a By means of glacial acetic acid* It was then found that every one of these fractions could be fractionated by means of glacial acetic acid into two fractions, the soluble and insoluble. The soluble fractions were freed from the acid by distillation under diminished pressure. The residue was redissolved in hot alcohol and on cooling gave a precipitate, this again could be fractionated by means of glacial acetic acid, so that finally the greatest part of the cerebroside could be crystallized out of glacial acetic acid.

*b By means of a mixture of one part of pyridine to one part of alcohol (98 per cent)* The precipitate obtained from the glacial acetic acid can be fractionated by this mixture. One part of the substance was dissolved in thirty parts of the mixture and allowed to stand over night at room temperature. The filtrate on cooling in an ice and alcohol mixture formed a second precipitate which was filtered off. The mother liquor from the second precipitate, evap-

orated to dryness at diminished pressure and recrystallized out of alcohol, gave a very small third precipitate. The fractionation by means of this solution was very sharp with the substance obtained from the cerebrine fraction. When the substance obtained from kersine was dissolved in the pyridine mixture the physical appearance of the two fractions was not very different one from the other, and the precipitate filtered with difficulty.

*Analysis of the substances obtained from the various fractions*

*Cerebrine out of alcohol*

(1a) 0.1359 gram of the substance gave 0.3442 gram of  $\text{CO}_2$  and 0.1388 gram  $\text{H}_2\text{O}$

(1b) 0.1304 gram of the substance gave 0.3324 gram of  $\text{CO}_2$  and 0.1326 gram  $\text{H}_2\text{O}$

(1a) 0.1920 gram of the cerebrine out of alcohol was employed for a Kjeldahl nitrogen estimation. It required for neutralization 2.5 cc of  $\frac{N}{10}$  acid.  $N = 1.9$  per cent

(1b) 0.2154 gram of cerebrine out of alcohol employed for a Kjeldahl nitrogen estimation required for neutralization 2.6 cc of  $\frac{N}{10}$  acid.  $N = 1.69$  per cent

*Cerebrine out of glacial acetic acid*

(2a) 0.1210 gram of the substance gave 0.3092 gram of  $\text{CO}_2$  and 0.1188 gram  $\text{H}_2\text{O}$

(2b) 0.1180 gram of the substance gave 0.2986 gram of  $\text{CO}_2$  and 0.1152 gram  $\text{H}_2\text{O}$

*Kersine out of alcohol*

(1) 0.1198 gram gave on combustion 0.3078 gram of  $\text{CO}_2$  and 0.1258 gram of  $\text{H}_2\text{O}$

(1) 0.1524 gram of the same substance employed for Kjeldahl nitrogen estimation required for neutralization 1.7 cc of  $\frac{N}{10}$  acid,  $N = 1.60$  per cent

*Kersine out of glacial acetic acid*

(2a) 0.1132 gram of the substance gave on combustion 0.2872 gram  $\text{CO}_2$  and 0.1168 gram  $\text{H}_2\text{O}$

(2b) 0.1102 gram of the substance gave on combustion 0.2836 gram  $\text{CO}_2$  and 0.1188 gram  $\text{H}_2\text{O}$

	C	H	N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated for cerebrin, $C_{48}H_{92}NO_8$	69 65	11 24	1 70
Found for cerebrine	1a 69 10	11 43	1 90
	1b 69 51	11 37	1 69
	2a 69 60	10 96	
	2b 69 00	10 92	
Found for kerasine (homocerebrine)	1a 69 90	11 75	1 60
	2a 69 20	11 55	
	2b 70 08	12 06	

*The melting points of the various cerebroside*

Regarding the melting points of the cerebroside the records of individual investigators show marked variations. The lowest value found by Parcus and by Kossel and Freytag for kerasine was 155–156°C, and the highest was recorded by Worner and Thierfelder for cerebrin, at 209–212°C. The melting points of our substances also varied, depending on the degree of purification of the substances. The cerebroside crystallized out of alcohol and, still containing some ash, showed decomposition rather than melting at the temperature of about 200°C. Substances carefully purified and, particularly, those purified by recrystallization out of glacial acetic acid possessed a very sharp melting point of 195°C. On fractionation out of the pyridine mixture two fractions were obtained—a more insoluble, melting at 210°C, and a less insoluble, melting at 195–198°C. The character of melting, described by Worner and Thierfelder for their cerebrin, leads us to the belief that the sample in their possession undoubtedly consisted of a mixture of the isomers.

*Optical rotation of the various fractions*

Two samples were employed for the determination of the optical activity of the cerebroside—one of the cerebrine and one of the kerasine (homocerebrine) fractions. They had been recrystallized from glacial acetic acid, and the analytical data from them have been reported in this communication. They had a sharp melting point, first liquefying and then decomposing at 195°C. In macroscopic appearance the first substance had the character of a fine granular powder, the other that of a solid waxy mass.



The optical rotation of the fractions was the following

*Cerebrine*

0.5295 gram of the substance was dissolved in 5 cc of pyridine. Total weight = 5.3249 grams and rotation at  $t = 25^{\circ}\text{C}$  and in pure yellow light in 1 dm tube was  $0.10^{\circ} (\pm 0.00)$  to the right

$$[\alpha]_D^{25} = +1.01^{\circ}$$

*Kerasine*

0.8175 gram of the substance was dissolved in 8 cc of pyridine. Total weight = 8.3755 grams. There was no optical activity detectible in a 1 dm tube and in pure yellow light

*Rotation of the substances fractionated by a pyridine alcohol mixture*

Five grams of each of the tested substances were dissolved in 30 cc of warm pyridine to which 120 cc of 98 per cent alcohol had been added. The solution was allowed to stand at room temperature ( $25^{\circ}\text{C}$ ) over night, then filtered on suction, washed with ether and dried. The mother liquor was chilled in an alcohol ice mixture and filtered at low temperature. The rotations of the fractions were the following

*Cerebrine*

First fraction 0.5090 gram of the substance dissolved in about 10 cc of pyridine. Total weight = 9.4852 grams. Rotation at  $25^{\circ}\text{C}$  in pure yellow light and in 2 dm tube =  $+0.20^{\circ}$

$$[\alpha]_D^{25} = +1.88^{\circ} (\pm 0.00^{\circ})$$

Out of 5 grams of the original substance about 2.5 grams of this first fraction and about 0.5 gram of a second fraction separated on cooling. The second fraction was inactive. The mother liquor from the second fraction gave a residue very soluble in alcohol. This was not tested further.

*Kerasine*

Originally inactive. The first fraction, or the one insoluble at room temperature, was optically tested. 0.4988 gram of the substance was dissolved in 10 cc of pyridine. Total weight = 9.4282 grams. Rotation in 2 dm tube, at room temperature of  $25^{\circ}\text{C}$ , and in pure yellow light =  $+0.05^{\circ} (\pm 0.00)$

$$[\alpha]_D^{25} = +0.47^{\circ} (\pm 0.00^{\circ})$$

The second fraction from the same sample was inactive

The values of the specific rotation of all these substances were lower than those reported by Thierfelder for cerebrin, hence one is led to the conclusion that they consisted of mixtures of isomers

*Hydrolysis of cerebrine for the purpose of estimating the proportion of galactose*

EXPERIMENT 1 Twenty grams of cerebrine were taken up in 500 cc of alcohol containing 50 grams of sulphuric acid and boiled in a water bath with return condenser for six hours Preliminary experiments have shown that the maximal hydrolysis cannot be obtained in a shorter time The solution was then placed in the refrigerator over night The fatty acids and esters were then removed by filtration The mother liquor was then diluted with one liter of water and boiled with return condenser five hours in a water bath The product of hydrolysis was then freed from sphingosine and from alcohol and the sugar estimated by reducing Fehling's solution, estimating the cuprous oxide by Volhard's method The yield of galactose was 5 grams or 20 per cent

EXPERIMENT 2 Three grams of cerebrine were taken up in 50 cc of alcohol containing 5 grams of sulphuric acid and treated in the same manner as in the preceding experiment The yield of galactose was 0.56 gram or 18.7 per cent

Theory for cerebrin, $C_{48}H_{93}NO_9$ , required	21.5 per cent of $C_6H_{12}O_6$
Found Experiment 1	20.0 per cent
Experiment 2	18.7 per cent

Taking into consideration that the removal of the fatty acid and of sphingosine always leads to some loss the agreement is quite satisfactory

*Analysis of the fatty acids*

The mixture of fatty acids and esters obtained on hydrolysis of the mixed cerebroside was saponified in the usual way and the soaps transformed into the free acids These were separated into two fractions by means of ether The fatty acids were dissolved in boiling ether and the ethereal solution allowed to stand in the refrigerator A fraction separated consisting practically of pure cerebronic acid The first precipitate thus obtained often had the

composition of C = 76.4, 76.3 or 76.1, and H = 12.6, 12.3 or 12.4. A comparatively small part of the total acids always remained in the ethereal mother liquor. The acids were obtained from this by removing the ether by distillation and recrystallizing the residue out of alcohol or out of acetone. The precipitate thus obtained varied in its carbon content from 77 to 78.5 per cent, and hydrogen content of about 12.5 to 13.0 per cent. It was thought that an acid of the composition  $C_{24}H_{48}O_2$  may be present in that fraction. However, in every experiment the acid value was low, and on the other hand, the fraction contained no methyl or ethyl esters, since the presence of methyl or ethyl groups could not be demonstrated by the method of Zeist and Fanto or by the saponification value.

In the later phases of the work it was learned that cerebronic acid formed a lithium salt, soluble in hot alcohol, and the same salt of  $C_{24}H_{48}O_2$  was insoluble. When the fraction containing the high carbon value was transformed into the lithium salt it was found that it was either all soluble in hot methyl alcohol or, if some remained as an apparently insoluble residue, it also was composed principally of cerebronic acid, containing carbon 76.5 per cent and hydrogen about 12.7 per cent. Thus the attempts to identify the acid  $C_{24}H_{48}O_2$  among the products of hydrolysis of mixed cerebrosides were not successful.

An experiment was then made in which 30 grams of cerebrine purified by glacial acetic acid were hydrolyzed in the usual way and the free acids were transformed into the lead salt and this again freed from lead. The analysis of the acid obtained in this manner had the following composition:

0.1194 gram of the substance gave 0.3292 gram CO<sub>2</sub> and 0.1350 gram H<sub>2</sub>O

	Calculated for $C_{23}H_{46}O_2$ (Cerebronic acid)	Found
C	75.38	75.40
H	12.56	12.65

Thus the attempts failed to discover on hydrolysis of mixed cerebrosides an acid that was not cerebronic acid. If such is present it is undoubtedly in the form of an impurity derived from some substance other than the cerebrosides.

# THE AMINO-ACID NITROGEN OF THE BLOOD

## PRELIMINARY EXPERIMENTS ON PROTEIN ASSIMILATION

By DONALD D. VAN SLYKE AND GUSTAV M. MEYER

(From the Rockefeller Institute for Medical Research, New York )

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Two opposing views are held concerning the manner in which the organism assimilates the amino-acids formed in the intestine from digested proteins

Abderhalden<sup>1</sup> believes that in passing the intestinal wall the amino-acids are synthesized into a blood protein. This enters the circulation, from which the cells of the tissues take it, break it down again into amino-acids and from these rebuild their own characteristic proteins. The reason for this view, that the amino-acids are resynthesized into protein while passing the intestinal wall, is, that although amino-acids are abundant in the intestinal contents, attempts to demonstrate them in normal blood have met with absolute failure. Against this view the objection is valid, that it is based on negative results and that more sensitive methods than those hitherto applied might reveal amino-acids in the blood.

Bugha<sup>2</sup> offers evidence indicating that the body is capable of utilizing amino-acids which have entered the blood stream. He injected intravenously into dogs amounts of amino-acid mixtures comparable to those formed from the food, and found that, when the injection was performed slowly, no serious ill effects followed and only a small part of the amino-acid nitrogen was excreted. Folin and Denis<sup>3</sup> injected large amounts of amino-acids directly into the small intestines of cats and noted a subsequent increase

<sup>1</sup> *Synthese der Zellbausteine*, 1912

<sup>2</sup> *Zeitschr. f. Biol.*, lvi, p. 162, 1912. Bugha also gives earlier literature.

<sup>3</sup> *This Journal*, vi, p. 87, 1912

- in the fraction of blood nitrogen left after subtracting the protein and urea. Neither the results of Buglia nor those of Folin and Denis, however, demonstrate the presence of amino-acids in the normal blood during periods of either digestive activity or rest. So long as this demonstration remains unperformed, the contention of Abderhalden cannot be refuted, that during normal digestion, with the chyme gradually passing in small portions from the stomach to the intestine and from the intestine to the blood, the latter passage is accompanied by synthesis of food amino-acids into blood protein.

It was evident that for the solution of the problem a method was necessary sufficiently delicate to ascertain decisively whether amino-acids are present in or absent from the blood, and sufficiently accurate to detect the fluctuations normally occurring in amino-acid nitrogen if it is present. We have found that the nitrous acid method for determination of amino nitrogen can be easily used for the blood and answers the above requirements. We have consequently applied it to the problem of protein assimilation.

#### METHOD FOR DETERMINATION OF AMINO-ACID NITROGEN IN BLOOD

Thirty to fifty cubic centimeters of freshly drawn blood are mixed with 9 or 10 volumes of 95 per cent alcohol to precipitate the proteins. The volume of the alcohol-blood mixture must be known, but in case it is not convenient to use a graduated cylinder for the mixture, its volume can be taken as the sum of the volumes of alcohol and blood without essentially affecting the results. The alcohol and blood are thoroughly mixed, the vessel containing them is closed, and twenty-four hours are allowed for precipitation of the proteins to become complete. The solution is filtered through a dry folded filter into a measuring cylinder, without washing the precipitate. The volume of filtrate obtained is noted, and is taken for analysis as an aliquot part of the total blood-alcohol mixture. The filtrate is then concentrated to a volume of 3-5 cc and used for determination of amino nitrogen by Van Slyke's nitrous acid method<sup>4</sup>. The use of a few drops of acrylic alcohol to prevent foaming is advisable.

<sup>4</sup> This *Journal*, ix, p 185, vii, p 275

The precipitation with alcohol is a simple and effective means of removing the proteins. The filtrate, even when concentrated, gives no biuret test. The completeness of the removal of proteins is further evidenced by the fact that when in control tests the alcohol was driven off from the filtrate and the residue was hydrolyzed with hydrochloric acid, the amino nitrogen set free by the hydrolysis amounted to only 2 mgm per 100 cc of blood.

Whether the alcoholic filtrate is concentrated on the water bath or under diminished pressure appears to make little difference with the results. We have, however, as a regular thing, concentrated under diminished pressure in a double-necked flask, transferring the solution towards the end of the distillation to a small flask, from which it can be removed with a minimum amount of wash water. This method of concentration is the most rapid, and the lipoids which separate when the alcohol is driven off take the form of a fine emulsion, which does not interfere mechanically or otherwise with the amino determination. They can be removed with ether, but this is unnecessary.

For the amino determination one can either wash the concentrated filtrate into a 10 cc graduated flask, measuring off 9.7 or 9.8 cc of this in the burette of the amino apparatus, or one can transfer the filtrate directly from the small distilling flask into the 10 cc burette of the amino apparatus, using several portions of 2-3 cc of water each to clean out the flask and wash the solution completely into the deaminizing bulb. We have usually used the latter method.

The amino determination is much simpler than in the case of urine, because the amount of ammonia in the blood is negligible, and the urea content is so slight that the correction for the proportion of urea nitrogen (about 3 per cent), given off while the amino-acids are quantitatively decomposed, is scarcely sufficient to affect the significance of results if it were entirely neglected. We have, however, always determined and made this correction according to the principle utilized in the determination of amino nitrogen in the urine.<sup>5</sup> The correction rests on the fact that in two to four minutes (according to the temperature) amino-acids give off 100 per cent of their nitrogen. Continuing

<sup>5</sup> This *Journal*, xii, p 275

the reaction for an equal length of time thereafter results, when urea is present, in the evolution of a further amount of nitrogen from the urea, this amount being equal to that evolved from the urea while the amino-acid was being decomposed in the first period. With the small amounts of urea present in the blood it is unnecessary to make an entire extra analysis to determine the correction. The latter is ascertained as follows. For the decomposition of the amino-acids the reaction is run with constant rapid shaking for four minutes at a temperature below  $20^{\circ}$ , for three and a half minutes at  $20-25^{\circ}$  and for two to three minutes above  $25^{\circ}$ , the time being accurately measured. At the end of the reaction the nitrogen gas is purified and measured in the usual way, and then expelled from the apparatus through cock *c* (see figure 2, p. 278, preceding number of this *Journal*). The solution left in the deaminizing bulb *D* is now shaken and treated exactly as described on page 280 of the above article for determining whether the reaction is complete or not. The length of time between the end of the first reaction and that of the second should be accurately that of the first reaction. The gas formed during the second period is shaken out with permanganate and measured. This represents the urea correction and is to be deducted from the first reading. The latter, minus this correction, represents only amino-acid nitrogen, since other forms of amino nitrogen, such as fatty amines, amino-purines, amino-pyrimidines and ammonia, react slowly and would if present be corrected for with the urea. In case it is inconvenient to finish the second reaction in as short a period as the first, it may be allowed to run one or two minutes longer, the correction being calculated on the rate of urea nitrogen evolved per minute. It is essential that the solution should be shaken rapidly during the last minute to expel all the nitrogen formed. The fact that under these conditions very nearly the same amount of nitrogen is evolved in the first and second periods is indicated by the following four pairs of determinations, each of which was made with 10 cc. of 0.5 per cent urea.

	I cc	II cc	III cc	IV cc
N in first three minutes	2 0	2 2	2 1	2 4
N in second three minutes	2 2	2 2	2 2	2 6

The maximum difference, 0.2 cc, is equivalent to only 0.1 mgm of amino nitrogen, and the corrections actually found in blood work are usually less than 2 cc

To ascertain whether the technique above described involves losses of amino-acids, particularly whether they are precipitated with or adsorbed by the proteins, the following experiment was performed

One hundred cc of fresh defibrinated blood, drawn from a dog which had fasted twenty-four hours, was precipitated with 1 liter of alcohol (Total volume = 1100 cc) Of the filtrate, 800 cc, equivalent to 72.7 cc of blood, were concentrated, freed from lipoids with ether and brought to 25 cc Ten cc portions, equivalent to 29.1 cc of blood, were used for amino determinations

I		II	
	cc		cc
N in first 4 minutes	2.95	N in first 3½ minutes	2.85
N in second 4 minutes	0.60	N in second 3½ minutes	0.50
N from amino-acids	<u>2.35</u>		<u>2.35</u>

2.35 cc of nitrogen at 26°, 754 mm, from 29.1 cc of blood indicate 4.4° mgm of amino-acid nitrogen per 100 cc

To another 100 cc of the same blood 1 gram of alanine, much more amino-acid than we have ever encountered in the blood in nature, was added Other details of the analysis were identical with the above

	I	II
N gas in three and one-half minutes	85.2 cc	85.6 cc
N from amino-acids and urea of blood	2.9 cc	2.9 cc
N from added alanine	82.3 cc	82.7 cc
Temperature	26°	27°
Pressure	754 mm	754 mm
Alanine N per 100 cc found	0.1554 gm	0.1550 gm
Alanine per 100 cc found	0.9880 gm	0.9860 gm
Alanine per 100 cc added	1.0000 gm	1.0000 gm

#### AMINO-ACID CONTENT OF BLOOD FROM NORMAL FASTING DOGS

The animals used for the following experiments had fasted for twenty to twenty-four hours before the blood was drawn<sup>6</sup> The results indicate that the amino-acid content varies within relatively narrow limits

<sup>6</sup> For the samples from the carotid artery and the vena cava we thank Dr Auer and Dr Githens



DOG NO	SOURCE OF BLOOD	VOL OF BLOOD EQUIVALENT TO FILTRATE USED	N (CORRECTED FOR UREA)	TEM- PERA- TURE	PRES- SURE	AMINO ACID NITROGEN PER 100 CC BLOOD
		cc	cc	degrees C	mm	mgm
1	Femoral artery	29 1	2 35	26	754	4 4
1	Femoral artery	29 1	2 35	26	754	4 4
2	Femoral artery	28 4	2 10	25	762	4 1
2	Femoral artery	28 4	2 20	25	762	4 3
3	Vena cava	21 5	2 10	24	760	5 4
4	Carotid artery	59 0	5 80	25	760	5 4
5	Femoral artery	51 0	4 20	25	760	3 7
6	Femoral artery	35 5	3 30	25	760	5 2
7	Carotid artery	37 5	2 20	31	758	3 1
8	Femoral artery	40 0	3 00	30	758	4 0
8	Mesenteric vein	40 0	2 90	30	758	3 9

*Experiment I Behavior of intravenously injected alanine*

A male dog of 14 kilos weight was catheterized and the bladder was thoroughly washed out. One cannula was placed in the right femoral vein, another in the left femoral artery. A sample of blood was drawn from the artery, and immediately afterwards the injection of 12 grams of *dl*-alanine dissolved in 400 cc of water into the vein of the other leg was commenced. At intervals samples of blood were drawn. At the end of the experiment the bladder was again washed out and the excreted amino-acid nitrogen determined. The results are here summarized.

	TIME	TIME AFTER END OF INJECTION	FREE AMINO-ACID N PER 100 CC	ESTIMATED ALANINE IN CIRCULATION	UNPRECIPITATED AMINO NITROGEN PER 100 CC FREED BY HYDROLYSIS
	p m	minutes	mgm	grams	mgm
Drew blood sample					
I Normal	3 15		4 2	0 00	2 1
Began injection	3 17				
Finished injection	3 30				
Drew sample II	3 35	5	37 2	1 47	1 8
Drew sample III	3 42	12	20 8	0 64	1 5
Drew sample IV	4 05	35	12 3	0 36	1 4
Drew sample V	4 30	60	12 3	0 36	1 7

Total nitrogen in urine	0 445 gram
Ammonia nitrogen in urine	0 015 gram
Free amino-acid nitrogen in urine	0 236 gram
equivalent to 1 501 grams alanine	
Total amino-acid nitrogen in urine	0 233 gram
The excreted alanine was optically inactive	

The injected alanine disappeared from the blood with such rapidity that five minutes after the injection only 1 47 grams out of the 12 grams injected remained in circulation and in thirty-five minutes only 0 36 gram. A relatively small proportion, 1 5 grams in all, was excreted, about 10 of the 12 grams *being almost immediately taken from the circulation by the tissues*. There was no evidence of synthetic processes in the blood, for the amino nitrogen freed by hydrolysis, which might be expected to increase if intermediate products of protein synthesis were present, remained practically constant.

The total amount of alanine in circulation was calculated on the assumption that the volume of the blood in liters is one-twentieth of the weight of the body in kilograms. While this is, of course, only a rough approximation, it suffices for the purpose of the experiment.

The details of the analyses follow

*Experiment I Determination of free amino-acid nitrogen Temperature 25°, pressure 762 mm*

SAMPLE	VOL OF SAMPLE	VOL OF BLOOD EQUIVALENT TO FILTRATE USED	N OAS EVOLVED IN 4 MIN	N OAS IN FOLLOWING 4 MIN	N OAS (CORR ) FROM AMINO-ACIDS	AMINO-ACID N PER 100 CC BLOOD
	cc	cc	cc	cc.	cc	mgm
I	{ 87	28 4	2 3	0 2	2 1	4 1
		28 4	2 4	0 2	2 2	4 3
II	{ 100	31 6	22 1	0 9	21 2	37 2
III	{ 76	24 0	9 3	0 4	8 9	20 7
		24 0	9 4	0 4	9 0	20 9
IV	{ 91	29 2	6 8	0 4	6 4	12 1
		29 2	7 0	0 4	6 6	12 5
V	{ 75	24 8	5 9	0 4	5 5	12 3
		24 8	5 8	0 3	5 5	12 3

For the above determinations each filtrate was brought to 25 cc, duplicate determinations being made on 10 cc portions of this.

Of the unused parts, 4 cc portions were used for hydrolysis. They were mixed with equal volumes of concentrated hydrochloric acid and

heated at 100° for twenty-four hours. The acid was evaporated off as completely as possible, and the ammonia removed by vacuum distillation with calcium hydrate. The latter was then dissolved with acetic acid and the sample used for amino determination.

*Experiment I Determinations of amino nitrogen after hydrolysis Temperature 27°, pressure 764 mm*

SAMPLE	VOL. OF BLOOD EQUIVALENT TO SAMPLE USED	N GAS IN 3 MIN	N GAS IN FOLLOWING 3 MIN	N GAS FROM AMINO- ACIDS	AMINO N PER 100 CC BLOOD	FREE AMINO N	AMINO N FREED BY HYDROL- YSIS
	cc	cc	cc	cc	mgm	mgm	mgm
I	11.4	1.50	0.10	1.40	6.3	4.2	2.1
II	12.6	9.10	0.20	8.90	39.0	37.2	1.8
III	9.6	4.50	0.20	4.30	22.3	20.8	1.5
IV	11.7	3.05	0.15	2.90	13.7	12.3	1.4
V	9.9	2.65	0.15	2.50	14.0	12.3	1.7

The urine voided during the hour after the beginning of the injection was made up to 100 cc. Portions of 2 cc. used for Kjeldahl determination required 6.40 and 6.30 cc. of  $\frac{N}{10}$  acid, the average indicating 0.445 gram of total nitrogen.

Fifty cubic centimeters were used for determination of ammonia and free amino nitrogen, as described by Levene and Van Slyke.<sup>7</sup> The ammonia neutralized 5.2 cc. of  $\frac{N}{10}$  HCl, indicating 0.0146 gram of ammonia N in the entire urine. The ammonia-free urine was brought back to 50 cc. volume and 10 cc. portions used for determination of free amino acid nitrogen.

I		II	
	cc		cc
N in first 3 minutes	45.0	N in first 4 minutes	45.2
N in second 3 minutes	1.9	N in second 4 minutes	2.2
Amino-acid N	43.1	Amino-acid N	43.0

These determinations were made at 27°, 758 mm. The average, 43.05 cc., indicates 0.236 gram of free amino-acid nitrogen in the total urine. This includes all the amino nitrogen, comparison with the following determination showing that no measurable amounts were conjugated.

For determination of the total amino nitrogen 40 cc. of urine were used, the sample being diluted to 50 cc. after removal of urea and ammonia. Ten cc. portions were used for amino determinations. They gave 33.5 and 33.4 cc. of nitrogen at 25°, 762 mm. indicating 0.233 gram of total amino nitrogen in the urine.

In order to determine whether the organism had destroyed or retained the natural component of the amino-acid (*d*-alanine)

<sup>7</sup> This Journal, xii, p. 275

and excreted the other (*l*-alanine), as found by Wohlgemuth<sup>8</sup> to be the case when certain *dl*-amino-acids were given *per os*, 25 cc of ammonia-free urine, containing 0.375 gram of alanine, were concentrated, and acidified with 0.5 cc of concentrated hydrochloric acid. The weight of the solution was 4.273 grams, the concentration of alanine hydrochloride 12.3 per cent. This, if all *l*-alanine, should have given a rotation of  $-1.2^\circ$  in a 1 dm tube. The observed rotation was only  $-0.04^\circ$ . The *dl*-alanine was, therefore, excreted practically unchanged and the alanine retained was equal parts dextro and levo.

### *Experiment II Absorption of alanine from the small intestine*

The animal used was a dog of 10 kilos weight which had fasted for twenty-four hours. Cannulas were placed in the left femoral artery and the mesenteric vein. Fifty cc samples of blood were drawn from each and diluted to 500 cc with alcohol. A loop of the small intestine was then ligated at both ends and 15 grams of alanine, dissolved in 100 cc of water, injected into it. After forty minutes, samples of blood were again drawn. The loop was then washed out, its contents diluted to 500 cc, and 10 cc used to determine the unabsorbed alanine nitrogen. The determination yielded 26 cc of nitrogen at  $26^\circ$ , 760 mm. From this

Total alanine N injected	grams 2.461
Alanine N unabsorbed	0.718
Alanine N absorbed	1.743

The blood analyses gave the following results. The temperature was  $30^\circ$ , barometer 758 mm.

SOURCE	VOL OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS EVOLVED IN 2.5 MIN	N GAS IN FOLLOWING 2.5 MIN	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC BLOOD
	cc	cc	cc	cc	mgm
Femoral artery be- fore injection*	40	3.7	0.7	3.0	4.0
Mesenteric vein be- fore injection	40	3.7	0.8	2.9	3.9
Mesenteric vein after injection	43	5.6	0.6	5.0	6.3

\* The determination on the blood from the femoral artery after the injection was lost.

<sup>8</sup> *Ber d deutsch chem Gesellsch*, LVIII, p 2064, 1904

The amount of amino-acid nitrogen in the mesenteric blood increased by 60 per cent as the result of the injection of alanine into the intestine. This, however, demonstrates only the possibility that amino-acids can pass the intestine. The sudden flooding of the intestine with a solution of one or more amino-acids is so entirely different from the gradual entrance of partially digested proteins from the stomach which occurs in normal digestion, that the results can not be utilized to explain the normal process of protein assimilation. The same restriction applies to the results of Fohn and Denis,<sup>9</sup> who flooded the intestines of cats with solutions containing unusual amounts of amino-acids and observed a subsequent rise in the fraction of blood nitrogen left after subtracting the urea and protein.

#### THE RISE OF THE AMINO-ACID CONTENT OF THE BLOOD DURING DIGESTION

##### *Experiment III*

We have, therefore, performed the following experiment. Samples of 50 cc of blood were drawn from the right femoral arteries of two dogs, of about 15 kilos weight each, which had fasted for

##### *Experiment III Effect of digestion on the amino-acid content of the blood*

SOURCE OF BLOOD	VOL OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS IN 3 5 MIN	N GAS IN FOLLOWING 3 5 MIN	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC BLOOD
<i>Dog A</i>	cc	cc	cc	cc	mgm
Right femoral artery before feeding	51 0	4 6	0 4	4 2	3 7
Mesenteric vein after feeding	38 3	8 3	1 5	6 8	9 5
Left femoral artery after feeding	37 7	7 2	1 2	6 0	8 6
<i>Dog B</i>					
Right femoral artery before feeding	35 5	4 0	0 7	3 3	5 2
Mesenteric vein after feeding	38 5	9 4	2 3	7 4	10 2
Left femoral artery after feeding	37 3	9 1	2 2	6 9	9 9

<sup>9</sup> This *Journal*, xi, p 87, 1912

twenty-four hours The dogs were in normal condition the next day, and each then devoured a kilo of fresh beef Five hours after the meal the animals were etherized and samples of blood drawn from both the left femoral artery and the mesenteric vein

The amino-acid content is about doubled during digestion It will be noticed that the correction for urea also increases This is to be expected from the results of Folin and Denis, who noted a marked rise in the urea content of the blood during digestion of protein

#### SUMMARY OF RESULTS

The gasometric method for direct determination of amino-acid nitrogen is easily applicable to blood from which the proteins have been precipitated by alcohol Duplicate results usually agree within 0.2 mgm of amino-acid nitrogen per 100 cc of blood

The blood of the dog normally contains amino-acid nitrogen The amount, in animals which have been fasting for twenty to twenty-four hours, is 3 to 5 mgms per 100 cc of blood

Twelve grams of alanine, injected during 13 minutes into the vein of a dog, were so rapidly removed from the blood stream that five minutes after the injection only 1.5 grams were left in the blood, and after 35 minutes but 0.4 gram Only 1.5 grams were excreted in the urine, the greater part of the injected amino-acid being evidently taken up by some of the tissues

Absorption of 10 grams of alanine from the small intestine increased the amino-acid nitrogen of the mesenteric blood from 3.9 to 6.3 mgm per 100 cc

During normal digestion of meat the amino-acid content of the blood undergoes a marked increase compared with its value before feeding It was doubled in the case of one dog and somewhat more than doubled in that of another The increase affected the blood from the femoral artery almost as much as that directly from the mesenteric vein

#### CONCLUSIONS

With the finding of amino-acid nitrogen in the normal blood the hypothesis, that the amino-acids formed in digestion are synthesized into blood protein while passing the intestinal wall,

becomes superfluous. The increase in amino-acid nitrogen of the blood, noted during digestion of protein, is, furthermore, positive evidence that amino-acids as such do normally pass the intestinal wall and enter directly into the blood current. The fact that the amino-acid content decreases but little during passage of the blood from the mesenteric vein out to the femoral artery indicates that the amino-acids are not held back or destroyed by the liver before reaching the other tissues. On the contrary, it seems that the amino-acids absorbed from the intestine circulate through the entire organism and are offered directly to the body cells in general. The fact that the amount of amino-acids normally present in the circulation is small, is accounted for by the rapidity with which the tissues take up amino-acids from the blood as soon as they become unusually abundant therein. This is illustrated by the disappearance of intravenously injected alanine from the blood stream.

The experiments here reported are preliminary to a more complete investigation of the problem of protein assimilation and of the effect of different physiological and pathological conditions upon the amino-acids of the blood.

# ON THE STRUCTURE OF THYMUS NUCLEIC ACID

BY P A LEVENE AND W A JACOBS

(*From the Rockefeller Institute for Medical Research, New York*)

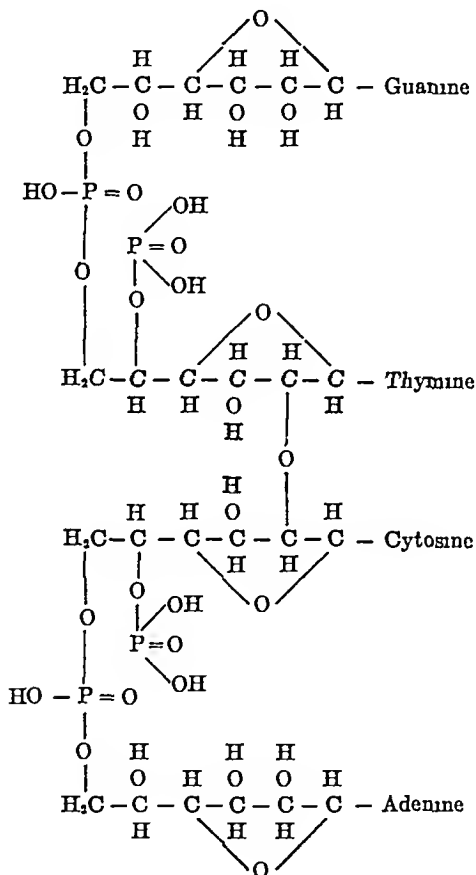
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Following the successful characterization of the inosinic and guanylic acids as phosphoric acid esters of purine ribosides, *z e*, simple nucleotides, and of the yeast nucleic acid as a complex of four such nucleotides, we at once attempted the same with the thymus nucleic acid. Evidence had already been obtained by Levene and Mandel<sup>1</sup> of the probable existence of a thymine nucleotide in this acid but owing to the meagre means at hand at that time for studying the product obtained the result required confirmation. From the very start our progress was greatly retarded by the marked differences shown by this acid towards the chemical treatment to which the ribose nucleotides had so readily yielded. The nucleic acid, when heated with ammonia in a sealed tube under the conditions for the preparation of the yeast nucleosides, remained apparently unchanged. When the temperature was raised to a point at which phosphoric acid was cleaved, free purines were also obtained. Likewise, when subjected to "neutral hydrolysis" the cleavage of phosphoric acid was always accompanied by the appearance of free purines and even pyrimidines. From the mixture no nucleosides could be isolated. This was finally accomplished by other methods which will be described elsewhere. We have been led to conclude that the instability of the sugar is the cause of the failure of chemical methods. Whereas the ribose nucleotides on hydrolysis with acids yield strongly reducing mixtures, the thymus nucleic acid shows but the weakest reduction of Fehling's solution after hydrolysis. That portion of the sugar which has been cleaved from combination is converted at once into levulinic acid. This was accomplished by boiling only two

<sup>1</sup> *Ber d deutsch chem Gesellsch*, vi, p 1905, 1908



hours with 2 per cent sulphuric acid Levene<sup>2</sup> has already pointed out that the glucosidic linking in this acid is much weaker than that in the yeast nucleic acid, as shown by the ready cleavage of all purines in the former by boiling in dilute acetic acid



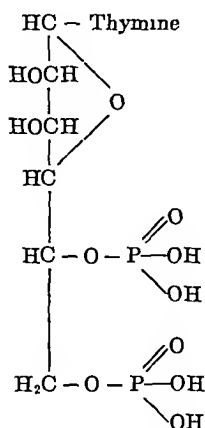
On the other hand the union of the phosphoric acid in the thymus nucleic acid resembles in stability that of the mosinic acid. Whereas by heating with dilute ammonia the phosphoric acid is most readily removed from the yeast and guanylic acids, under the same conditions both the mosinic and thymus nucleic

<sup>2</sup> *Biochem Zeitschr*, x, p 215 1903

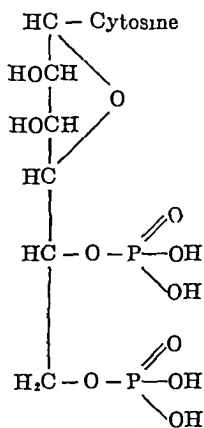
acids remain unchanged. The inosinic acid is decomposed only by neutral hydrolysis, a condition under which unfortunately the glucosides of the thymus are destroyed. This stability, together with the stability of the pyrimidine glucosides which is analogous in every respect to the conditions found in the yeast nucleic acid, has enabled us to obtain by partial hydrolysis with dilute acid intermediary products from which we have been able to construct a formula representing in all probability the structure of the acid.

It has been assumed by many that the nucleus of the nucleic acid is a polyphosphoric acid in which the phosphoric acid molecules are linked together in an anhydride form. As is well known, the acid itself is stable towards alkali, a property not possessed by anhydrides, so that *a priori* such a structure is unlikely. As a matter of fact, the products formed upon hydrolysis of the acid are incompatible with such a mode of linking. As developed by the present investigation, the nucleic acid is represented by the formula on preceding page.

The two pyrimidine nucleotides are joined together presumably by an ether linking between the sugars. Each sugar of the thymine and cytosine nucleosides is conjugated with a secondary phosphoric acid and then again with a tertiary phosphoric acid which in turn forms a bridge between the pyrimidine and the



Hexo-thymidine diphosphoric acid

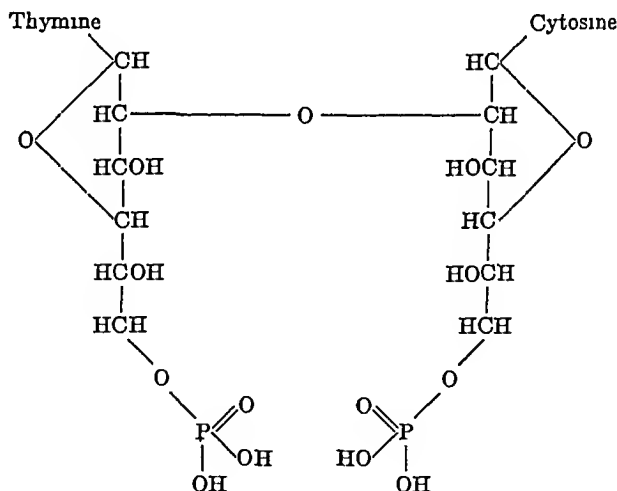


Hexo-cytidine diphosphoric acid

purine nucleosides This view is based upon the following facts Upon hydrolysis of the nucleic acid with 2 per cent sulphuric acid for two hours the purines are completely removed and the sugar originally in union with them is almost completely converted into levulinic acid This was readily obtained by extraction with ether and identified by the hydrazid and silver salt The resulting mixture, as described below, was fractionated with phosphotungstic acid From the portion not precipitated by this reagent the crystalline brucine salts and barium salts of a hexocytidine diphosphoric acid and a hexo-thymidine diphosphoric acid were obtained of the structure given on preceding page

Though not yet proven, we consider this position of the phosphoric acids on the sugars probable, because of the great stability shown by them, a condition analogous to that found in inosinic acid

From the phosphotungstic fraction we have succeeded in isolating a dinucleotide of the following structure



In all these formulae no significance is to be attached to the configuration of the sugar since its nature has not yet been determined

The crystalline brucine salt and barium salt of this substance were also prepared and from the barium content shown by the

analysis we must conclude that each phosphoric acid contains a secondary and a tertiary hydroxyl and the linking between the two nucleotides occurs between the sugars as shown above. By fractionation with mercury a mixture of the barium salts of the simple thymine and cytosine nucleotides was obtained. The experimental part regarding both the dinucleotide and the simple nucleotides will be described in a subsequent communication.

#### EXPERIMENTAL PART

##### *Hydrolysis of the nucleic acid*

Two hundred grams of nucleic acid (from fish sperm) were heated on the water bath in 3 liters of 2 per cent sulphuric acid until solution was complete and then boiled for two hours under a reflux condenser. After cooling silver oxide was added as long as a precipitate of purine silver separated and the mixture was allowed to stand over night. The filtrate from the purine precipitate was then treated further with silver oxide until a drop of the solution added to dilute sodium hydrate gave a precipitate of silver oxide. To this solution a warm saturated solution of barium hydrate was added until just alkaline to litmus. The precipitate which contained the silver and barium compounds of the nucleotides, phosphoric acid and free pyrimidines, was rapidly filtered by suction and washed well with water. The filtrate after removal of silver with hydrogen sulphide gave a considerable test with orcin and copper, and slightly reduced Fehling's solution. After removal of barium from this with sulphuric acid and concentration to a small bulk we were able to precipitate with basic lead acetate and barium hydrate a small amount of material from which a barium salt was prepared. This salt reduced Fehling's solution and contained conjugated phosphoric acid. The salt so obtained is presumably the barium salt of the hexose phosphoric acid contained in the nucleic acid. The quantity of substance which was still quite impure was too small to purify further.

The above silver barium precipitate was decomposed by suspending in a slight excess of dilute sulphuric acid and passing hydrogen sulphide through the mixture. After complete decomposition the filtrate was freed from hydrogen sulphide and precipitated by mercuric sulphate solution. The heavy

of the mercury salt of the mixed nucleotides was filtered and well washed. In the filtrate are contained besides free phosphoric acid the simple nucleotides which were isolated by a method to be described later. The above mercury precipitate was suspended in water and completely decomposed by hydrogen sulphide. The mixture was neutralized with pure barium carbonate and then acidified with acetic acid to keep in solution some of the nucleotides. The filtrate, after concentration to a small volume, was precipitated by several volumes of alcohol. The heavy precipitate of barium salts was filtered, washed with 70 per cent alcohol, then with absolute alcohol and ether and then dried. The yield varied from 50 to 75 grams. The mixture so obtained was dissolved in about 5-10 parts water and the barium removed with a slight excess of sulphuric acid. To the filtrate 50 per cent sulphuric acid was added until the solution contained 10 per cent acid. To this concentrated phosphotungstic acid was added until precipitation was complete. The phosphotungstate fell as a heavy gummy mass to the bottom and after standing a while at 20° the clear supernatant liquor was poured off. The phosphotungstate was again dissolved in several volumes of hot water and again precipitated by addition of sulphuric acid to 10 per cent. The phosphotungstate contained the dinucleotide fraction. The two mother liquors were joined and the phosphotungstic acid shaken out with amyl alcohol. The solution was then treated with barium hydrate solution until just alkaline to phenolphthalein, again acidified with acetic acid, filtered and concentrated to several hundred cubic centimeters. For further purification the nucleotides were precipitated again with mercuric sulphate and the mercuric precipitate decomposed with hydrogen sulphide. From the filtrate, after removal of the hydrogen sulphide, a trace of sulphuric acid was removed quantitatively with barium hydrate. The acidity of the solution was then determined by titration and an equivalent amount of commercial brucine dissolved in alcohol was added to the mixture. Though crystallization may begin at once, the whole mixture was concentrated *in vacuo* to dryness and the residue dissolved in a sufficient quantity of hot 85 per cent alcohol. On cooling the mixture crystallized to a solid mass. After standing twenty-four hours it was broken up with a glass rod, filtered and washed with 75 per cent alcohol. The brucine

salt so obtained is the salt of the hexo-thymidine diphosphoric acid After several recrystallizations from hot 85 per cent alcohol it is pure

*Hexo-thymidine diphosphoric acid*

As so prepared the neutral or tetrabasic salt crystallizes in variegated aggregates of long microscopic plates When rapidly heated in a capillary tube it sinters at about 172° The acid salts crystallize in globular aggregates of microscopic needles of higher melting point The neutral salt is practically insoluble in cold water, absolute alcohol, acetone, chloroform and the other neutral organic solvents In hot dilute alcohol and hot dilute acetone it is fairly easily soluble

For analysis it was dried in vacuum over phosphorus pentoxide at 110°

0.3358 gram substance gave 0.0375 gram  $Mg_2P_2O_7$

P	Calculated for		Found
	$C_{11}H_{13}N_5O_{11}P_2$	$4C_{11}H_{13}N_5O_{11}$	
	3.06		3.11

For conversion into the barium salt the brucine salt was suspended in water in a separatory funnel, an excess of ammonia added and shaken out several times with chloroform The aqueous solution of the ammonium salt was placed in a distilling flask, a few drops of phenolphthalein added, and distilled *in vacuo*, the receiving flask containing dilute sulphuric acid During the distillation a saturated solution of pure barium hydrate was added drop by drop until the solution remained on continued distillation just alkaline to the phenolphthalein After the removal of the ammonia by this process the barium salt, which had already partly separated upon the first addition of barium hydrate, was brought just into solution by the addition of a few drops of acetic acid, an excess being avoided The solution was then brought to a boil The barium salt which is more insoluble in hot than in cold water separated as a white powder, which under the microscope appeared as globules in which crystal formation was hard to discern The salt was filtered hot, washed with hot water, then with alcohol and ether and dried So prepared the salt is pure for analysis For the analysis it was dried *in vacuo* over

phosphorus pentoxide at 110° Many samples obtained in different experiments were analyzed

0 1879 gram substance gave 0 1248 gram CO<sub>2</sub>, 0 0360 gram H<sub>2</sub>O  
 0 1579 gram substance gave 0 0514 gram Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>  
 0 1512 gram substance gave 0 0990 gram BaSO<sub>4</sub>  
 0 1397 gram substance gave 0 00574 gram N (Kjeldahl)  
 0 2425 gram substance gave 0 0101 gram N (Kjeldahl)

	Calculated for C <sub>11</sub> H <sub>14</sub> O <sub>12</sub> N <sub>2</sub> P <sub>2</sub> Ba <sub>2</sub>	Found
C	18 44	18 12
H	1 96	2 13
		I II
N	3 91	4 11 4 18
P	8 66	9 06
Ba	38 30	38 54

For the optical determination 0 346 gram substance was dissolved in 5 cc of  $\frac{N}{3}$  HCl Total weight of solution, 5 533 grams In 2 dm tube at 30° with D-light it rotated 0 84° to the right Calculating the specific rotation of the free acid without regard to the specific gravity,

$$[\alpha]_D^{30} = +10.86^\circ$$

When the strong acetic acid solution of this salt is concentrated slowly on the water bath it gradually separates as a white crust which under the microscope is seen to consist of aggregates of colorless needles When exposed, the tertiary salt absorbs carbon dioxide from the air

The free acid was not prepared crystalline The aqueous solution of the free acid is precipitated by mercury salts, except the chloride, by ammonium molybdate and lead acetate as well as by other heavy metals Phosphotungstic acid precipitates it as an oil in concentrated solutions when sulphuric acid is added to 20–25 per cent This precipitate is soluble in water and precipitated by the addition of strong sulphuric acid In following the method given above for the removal of the phosphotungstate fraction containing the dinucleotide, the sulphuric acid content of the mixture must not exceed 10 per cent to avoid precipitation of this thymine complex as well as the cytosine complex described below

Upon hydrolysis of this acid with 10 per cent sulphuric acid in a sealed tube at 125° for three hours the phosphorus is com-

pletely broken off From the mixture thymine was obtained in typical form and also levulinic acid

With orcin and a drop of copper chloride solution the characteristic test for hexoses is obtained, but the green color is of considerably less intensity than that obtained with a hexose or with the original nucleic acid This is in harmony with the experience obtained with the pyrimidine ribosides obtained from the yeast nucleic acid As seen in the method of preparation the union of the pyrimidine with the sugar is comparatively stable, and no reducing substances are obtained upon hydrolysis Also bromine water added to the complex is instantly decolorized and the resulting solution reduces Fehling's solution Under certain conditions hydrogen with colloidal palladium was found to reduce the compound The resulting solution gave a very strong orcin test and upon hydrolysis yielded a strongly reducing solution containing a phospho-sugar and a dehydro-thymine From these facts which agree with the experience obtained by Levene and Laforge<sup>3</sup> with cytidine and uridine from the yeast nucleic acid we conclude that the union between pyrimidine and sugar is of the same nature in the two nucleic acids, *i e*, a simple glucosidic union

#### *Hexo-cytidine diphosphoric acid*

The mother liquors obtained from the above brucine salt when concentrated to small volume yield, on standing, more of the salt of the thymine complex When this is filtered and the mother liquors further concentrated and treated with absolute alcohol to permanent turbidity, on standing several days, the neutral brucine salt of the analogous cytosine complex separates in long plates with pointed ends which can be easily seen by the naked eye After filtration the salt is dissolved in hot 95 per cent alcohol On standing it separates as large prisms After twenty-four hours the crystallization is complete This salt is more beautiful than the thymine salt It is likewise much more soluble in warm alcohol, even in hot absolute alcohol in which the thymine complex is very little soluble

For the analysis it was again recrystallized and dried *in vacuo* over phosphorus pentoxide at 110°

<sup>3</sup> *Ber d deutsch chem Gesellsch*, xlv, pp 608 20



0.2839 gram substance gave 0.0363 gram  $Mg_2P_2O_7$

0.4878 gram substance gave 33.2 cc N (29°, 767 mm)

	Calculated for $C_{10}H_{17}N_3O_{12}P_2$	Calculated for $4C_{12}H_{23}N_3O_4$	Found
P	3.09		3.56
N	7.66		7.46

For the preparation of the barium salt the method described above for the thymine complex was used. It is also more insoluble in hot water than in cold water and advantage was taken of this fact for the preparation of the pure neutral salt. For analysis it was dried *in vacuo* over phosphorus pentoxide at 110°

0.1922 gram substance gave 0.1203 gram  $CO_2$ , 0.0430 gram  $H_2O$

0.1484 gram substance gave 0.0085 gram N (Kjeldahl)

0.1877 gram substance gave 0.0107 gram N (Kjeldahl)

0.3681 gram substance gave 5.25 cc amino N (22.5°, 752 mm)

0.1730 gram substance gave 0.1162 gram  $BaSO_4$

0.1507 gram substance gave 0.0493 gram  $Mg_2P_2O_7$

	Calculated for $C_{10}H_{17}N_3O_{12}P_2Ba$	Found
C	17.08	17.06
H	1.85	2.49
N	5.96	5.71
Amino N	1.99	1.59
P	8.82	9.11
Ba	39.00	39.55

The salt was obtained only as an amorphous powder. For the optical determination 0.3793 gram dried substance was dissolved in 4 cc N hydrochloric acid solution. Total weight of solution, 4.427 grams. In a 2 dm tube with D-light it rotated at 25°, 3.29° to the right. Calculating for the free acid and without regard for the specific gravity,

$$[\alpha]_D^{25} = +31.45^\circ$$

The free acid resembled closely in its behavior that of the thymine complex toward precipitants and reagents. It is likewise not more easily precipitated by phosphotungstic acid. The difference in solubility of the brucine salts is the only method of which we now know which will accomplish a separation of the two acids.

From this acid levulinic acid and cytosine were obtained. The latter was analyzed as the picrate.

0.1403 gram substance gave 30.9 cc N (27°, 764 mm)

	Calculated for $C_8H_7N_3O$	Calculated for $C_8H_7(NO_2)_3OH$	Found
N	24.62		24.32

# ON GUANYLIC ACID

## SECOND PAPER <sup>1</sup>

BY P A LEVENE AND W A JACOBS

(From the Rockefeller Institute for Medical Research, New York)

(Received for publication, July 19, 1912)

Guanylic acid undoubtedly belongs to the group of simpler substances of that class. Its molecule contains only one base, guanine, and one carbohydrate, *d*-ribose. These two substances are combined through a glycosidic linking as guanosine. There is, however, still a lack of agreement in the minds of individual investigators as to the degree of its simplicity. The latest publication on the subject is that by Bang<sup>2</sup> in 1910. In this article the author attacks with great ardor and considerable bitterness the view of those workers who regard guanylic acid as a simple nucleotide. According to Bang, guanylic acid in its structure is analogous to thymus nucleic and not to inosinic acid. The very lengthy argument of the author is based on the results of the ultimate analysis of various amorphous salts of guanylic acid and on the property of the salts to gelatinize. However, guanosine—a simple guanine-pentoside—shares with guanylic acid the property of gelatinizing when it contains only a slight proportion of mineral impurity. Also the argument of the elementary analysis is not very convincing for the reason that in the publication of Bang no evidence was offered to prove the purity of the analyzed acids. It is true that the opposing investigators have not yet succeeded in presenting analytical data of such a nature as to render their contentions absolutely convincing.

Several years ago the method of preparation of the acid was considerably simplified by us and we had hoped to be in a position

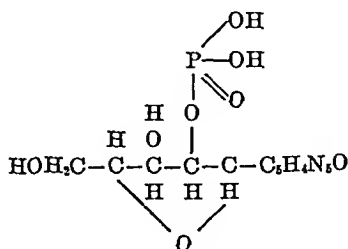
<sup>1</sup> *Ber d deutsch chem Gesellsch*, xlii, p 2469, 1909

<sup>2</sup> *Biochem Zeitschr*, xxi, p 293, 1910

to obtain the substance in that degree of purity which would permit us to clear up the disputed points in the structure of the substance. Pressure of other work caused the delay of the undertaking and only very recently have we been in a position to again direct our efforts to the work on guanylic acid.

It is now possible to obtain the substance in the form of a pure crystalline brucine salt. The process that led to the preparation of this salt was complex. The acid was originally obtained directly from pancreas glands as an impure lead salt. This was transformed into an acid sodium salt. The latter salt was further purified as a mercury salt. The advantage of the last purification lay in the fact that a solution of mercuric sulphate forms a mercuric salt of guanylic acid insoluble in dilute acids. Thus a means is given by which all other bases may be removed from the mercuric salt. A solution of the free acid can be easily obtained from the mercuric salt. In that condition guanylic acid showed no tendency to gelatinize. The acid in this solution was easily transformed into the crystalline brucine salt and was analyzed as such. The brucine salt was then transformed into the neutral barium salt and this again analyzed. The analysis of the two last named salts permitted us to formulate the composition of guanylic acid as  $C_{10}H_{14}N_5O_8P$  or as a mononucleotide. As a polynucleotide is formed through the process of anhydride formation, the polynucleotide hypothesis of the structure of guanylic acid requires values of carbon, nitrogen and phosphorus higher than those actually found.

On the basis of this the following constitution may be ascribed to guanylic acid



The substance is optically active, showing  $[\alpha]_D^{20} = -1.27^\circ$  in hydrochloric acid solution.

There is some basis for the assumption that the structure of guanylic acid is not identical with that of inosinic acid. There apparently exists a difference in the union between phosphoric acid and the carbohydrate in these acids analogous to the difference in the union between phosphoric acid and the nucleosides of the two more complex acids, namely, that of the thymus and of the yeast nucleic acid. Both guanylic and yeast nucleic acid permit the detaching of phosphoric acid quite readily, whereas in the inosinic and in the thymus nucleic acid the same reaction is accomplished with great difficulty. This difference is probably due to a difference in the position of the phosphoric acid on the sugar. When hydrolyzed under the same conditions employed for the preparation of ribose phosphoric acid from inosinic acid, the phosphoric acid was completely cleaved from guanylic acid. We have, therefore, as yet been unable to obtain definite evidence regarding the position of the phosphoric acid on the sugar.

#### *Preparation of the crude guanylic acid*

The glands were trimmed and passed through a hashing machine. They were suspended in water, which was brought to a boil and to the mixture was added potassium acetate in substance enough to make the concentration 5 per cent.

When the mixture cooled down to about 65° enough sodium hydrate was added to make its concentration 5 per cent. This mixture was allowed to stand over night, when it was neutralized by means of picric and acetic acids as long as a precipitate formed. The precipitate was then removed by filtration and to the filtrate a 25 per cent solution of neutral lead acetate was added. The mixture was brought to a boil and filtered. To the filtrate more lead acetate and ammonia were added as long as a precipitate formed. This precipitate contained guanylic acid and guanosine. To obtain the guanylic acid the substance was suspended in hot water and transferred to flasks immersed in a hot water bath. A stream of hydrogen sulphide was passed through the mixture. The lead sulphide was ultimately removed by filtration and the filtrate concentrated to small volume. It was then rendered alkaline by means of ammonia and precipitated with a large excess of 95 per cent alcohol. This was done in order to remove the

guanosine which remained in the solution, the mixed ammonium and sodium salts of guanylic acid remaining in the precipitate. The precipitate was then dissolved in hot water and placed in the refrigerator where the acid salt of guanylic acid settled out as a gelatinous mass which was filterable without much difficulty.

In the early part of the work attempts were made to obtain crystalline sodium salts of this substance. The experiments were in a way successful but were later abandoned when a more convenient method for purification of the crude guanylic acid was devised.

#### *Purification of the crude guanylic acid*

The crude guanylic acid was suspended in water and sulphuric acid added until solution was complete. A solution of mercuric sulphate was then added as long as a precipitate formed. This was filtered and washed well with hot water. The precipitate was well suspended in water and completely decomposed by hydrogen sulphide. The mercuric sulphide was filtered off and a slight trace of free sulphuric acid in the filtrate removed quantitatively with barium hydrate. The solution so prepared, containing the practically pure acid, contrary to that of the crude material, does not gelatinize. The addition of small quantities of alkali causes gelatinization. For isolation the acid was first converted into the brucine salt.

#### *Brucine salt of guanylic acid*

For this purpose the acidity of the solution was determined by titration and an equivalent amount of commercial brucine dissolved in a little alcohol was added. Crystallization began at once. Under the microscope the salt appeared as rosettes of thin colorless rectangular plates. On longer standing in the ice box more of the salt crystallized in long thin plates which were visible to the naked eye. The salt was filtered and dried.

It is very difficultly soluble in cold water, alcohol and other neutral organic solvents. It is more soluble in hot water and hot dilute alcohol. Thirty per cent alcohol is best for recrystallization. It crystallizes completely from this only on long standing. So

prepared it is pure for analysis For analysis it was dried in vacuum over phosphorus pentoxide at  $110^{\circ}$

0.2852 gram substance gave 29.6 cc N at  $31^{\circ}$ , 756 mm

0.3102 gram substance gave 0.0296 gram  $Mg_2P_2O_7$

	Calculated for $C_{10}H_{14}N_5O_5P \cdot 2C_{23}H_{25}N_2O_4$	Found
N	10.94	11.00
P	2.69	2.65

### *Barium salt of guanylic acid*

For further characterization the barium salt was prepared For this purpose the ammonium chloroform method described in the article on the thymus nucleic acid was employed The aqueous solution of the ammonium salt was then converted by distillation with barium hydrate into the barium salt The barium salt remained as a difficultly soluble residue When the barium was over added a basic salt was formed which analysis showed to have approximately two molecules of barium

The second barium is probably contained in the guanine nucleus since this is known to form a barium salt This basic salt is more soluble in water than the neutral salt and reacts strongly alkaline The difficulty of obtaining this pure caused us to prepare the neutral salt For this purpose the above mixture was freed quantitatively from barium with sulphuric acid and the clear filtrate obtained treated with pure barium hydrate solution until just neutral to phenolphthalein The neutral barium salt settled at once as a difficultly soluble white amorphous powder It was filtered, washed rapidly with water, then successively with alcohol and ether and dried

For the analysis it was dried *in vacuo* over phosphorus pentoxide at  $110^{\circ}$

0.2218 gram substance gave 0.1944 gram  $CO_2$ , 0.0559 gram  $H_2O$

0.2286 gram substance gave 0.0321 gram N (Kjeldahl)

0.2367 gram substance gave 0.0333 gram N (Kjeldahl)

0.2257 gram substance gave 0.0492 gram  $Mg_2P_2O_7$

0.2119 gram substance gave 0.0935 gram ash ( $Ba_2P_2O_7$ )

	Calculated for $C_{10}H_1 N_5O_5PBa$	Found	
C	24 10	23 91	
H	2 41	2 80	
N	14 06	I 14 06	II 14 05
P	6 22	6 07	
Ba <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	44 98	44 12	

When exposed to the air it absorbs carbon dioxide

For the optical determination of the pure guanylic acid the barium salt was dissolved in acid 0.3075 gram dry substance was dissolved in 4 cc of N HCl Total weight of the solution, 4.347 grams In a 2 dm tube with D-light at 25° it rotated 0.13° to the left Calculating for the free acid and without regard for the specific gravity,

$$[\alpha]_D^{25} = -1.27^\circ$$

## ON THE PREPARATION OF GLUCOSIDES

By W. A. JACOBS

(From the Rockefeller Institute for Medical Research, New York)

(Received for publication, July 19, 1912)

For the preparation of the glucosides of sugars two general methods have been devised by Fischer<sup>1</sup>. In one, the alcoholic solution or suspension of the sugar is saturated with hydrochloric acid. From the resulting mixture the hydrochloric acid is neutralized with barium carbonate and removed as barium chloride. But the great expenditure of time and labor required by the repeated concentrations and extractions with alcohol led Fischer to devise a second method<sup>2</sup>. In this the sugar and alcohol are heated with a small amount of dry hydrochloric acid for thirty to fifty hours and the small amount of hydrochloric acid removed by silver oxide. In this method the reaction is not complete and moreover requires several days.

In the work which required the preparation of large quantities of these compounds, we were led to a simple modification of Fischer's first method which enabled us to prepare the glucosides within a day and in good yield. This device was used in one instance by Fischer himself,<sup>3</sup> but was not recommended as a general method. As it may be found of service to other workers the method is described here in detail.

The solution or suspension of one part of the powdered sugar in ten parts of the dry alcohol is saturated with hydrochloric acid with cooling as in Fischer's first method. After one hour all reducing power disappears. The concentrated to one-fourth its volume *in vacuo* at 20°

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.*, **xxvi**, p. 2400

<sup>2</sup> *Ibid.*, **xxviii**, p. 1145

<sup>3</sup> *Ibid.*, **xxvii**, p. 2484



poured into ordinary alcohol containing a few cubic centimeters of acetic acid. The excess of hydrochloric acid is removed by adding a fine suspension of pure lead carbonate in a little water until the mixture no longer reacts acid to congo paper. The filtrate, after treatment with hydrogen sulphide, is then concentrated *in vacuo* and the glucosides isolated as usual.

# A NOTE ON THE REMOVAL OF PHOSPHOTUNGSTIC ACID FROM AQUEOUS SOLUTIONS

By W A JACOBS

*(From the Rockefeller Institute for Medical Research, New York )*

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For the removal of the excess of phosphotungstic acid from the mother liquor, after the precipitation of phosphotungstates from aqueous solutions, the usual procedure is precipitation with barium hydrate. The precipitate formed is generally very voluminous and where the mother liquor contains valuable material the loss through adsorption is apt to be great. Winterstem has already pointed out that this difficulty may be overcome by shaking out the solution with ether. In this method a mechanical difficulty enters since the solution separates into three layers, the lowest being an oily layer of ether dissolved in phosphotungstic acid, a middle aqueous layer and a top ethereal layer. The lowest layer disappears after several shakings but the operation must still be repeated many times and complete removal of the phosphotungstic acid is not assured. In amyl alcohol was found a means of quickly attaining this end. The partition coefficient of phosphotungstic acid between water and amyl alcohol is enormously in favor of the latter so that if sufficient amyl alcohol is employed after allowing time for complete separation of the layers practically all the phosphotungstic acid is removed by one shaking. If the alcohol is added in small portions the amyl alcohol phosphotungstic mixture settles as an oil. When this is repeated with fresh amyl alcohol once or twice, depending upon the amount of phosphotungstic acid present, the amyl alcohol finally floats on the top. The phosphotungstic acid is then completely removed from the aqueous solution.

When this method is to be employed one must be assured that the substances sought in the aqueous solution are not soluble in

amyl alcohol If this is the case, the addition of ether to the amyl alcohol, even to 80 per cent, may reduce this solubility without impairing the usefulness of the alcohol as an extracting agent for phosphotungstic acid In some cases where phosphotungstates themselves are soluble in hot or cold water we have been able to decompose them by this means with a great saving of time, labor and material

# PROTEIN METABOLISM IN EXPERIMENTAL DIABETES

By A. I. RINGER

(From the Department of Physiological Chemistry of the University of Pennsylvania)

(Received for publication, July 27, 1912)

## I

One of the most striking phenomena in experimental diabetes is the very marked rise in the protein catabolism. Minkowski,<sup>1</sup> Hedon<sup>2</sup> and Falta<sup>3</sup> called attention to it in cases of depancreatized animals. Lusk<sup>4</sup> and his pupils showed it to exist in phlorhizin glycosuria. The increase in the protein catabolism above the starvation requirements has been found by Lusk to be from 400 to 560 per cent in dogs and 238 per cent in a goat.

In pancreatic diabetes as well as in phlorhizin glycosuria, the amount of glucose in the urine bears a fairly constant relationship to the nitrogen. Depancreatized dogs yield approximately 2.8 grams of glucose to every gram of nitrogen. Phlorhizinized herbivorous animals eliminate glucose and nitrogen in approximately the same ratio. Dogs, however, present a much higher ratio, 3.6 to 1.

From experiments by Lusk,<sup>5</sup> Arteaga,<sup>6</sup> Cremer<sup>7</sup> and others, we note that in herbivorous animals, during phlorhizin diabetes, the protein metabolism does not rise as much above the starvation requirements as it does in the dog, which yields a higher D/N ratio.

<sup>1</sup> Minkowski *Arch f exp Pathol u Pharm*, **xxi**, p 149, 1893

<sup>2</sup> Hedon *Arch d Physiol*, 5th series, **iv**, p 258, 1892

<sup>3</sup> Falta, Grote und Stähelin *Beitr z chem Physiol u Path*, **v**, p 199, 1907

<sup>4</sup> Graham Lusk *Zeitschr f Biol*, **xxvi**, p 82, 1898, Reily, Nolan and Lusk *Amer Journ of Physiol*, **i**, p 397, 1898

<sup>5</sup> Lusk *loc cit*

<sup>6</sup> Arteaga *Amer Journ of Physiol*, **vi**, p 173, 1901

<sup>7</sup> Cremer *Münch med Wochenschr*, **xl**, p 274, 1893

In the experiments reported below an attempt was made to find the factors that govern the protein metabolism in phlorhizin glycosuria

TABLE I  
*Twenty-four-hour periods*

PERIOD	WEIGHT	TOTAL NITROOEN	NITROOEN PER HOUR	TOTAL GLUCOSE	OLUCOSE PER HOUR	D N	REMARKS
I	11 92	2 66	0 111				Fasting
II	11 28	6 63	0 276	34 61	1 44	5 22	Phlorhizin
III	10 70	8 12	0 340	34 64	1 44	4 27	Phlorhizin
IV	10 40	9 68	0 403	34 94	1 46	3 60	Phlorhizin
V	10 00	8 47	0 353	31 68	1 32	3 74	Phlorhizin
VI	9 75	9 64	0 402	31 44	1 31	3 26	Phlorhizin
VII	9 40	7 46	0 311	25 92	1 08	3 47	Phlorhizin

The results in table I represent the course of a typical phlorhizin experiment. It is one record of a great many. The animal was allowed to fast for three days prior to the administration of the phlorhizin. Period I is the third fasting day. Beginning with the morning of the second period, phlorhizin in two-gram doses, dissolved in 25 cc. of warm 1.5 per cent  $\text{Na}_2\text{CO}_3$  solution was administered subcutaneously three times per day. The animal fasted throughout the course of the entire experiment.

During the first period the animal eliminated 2.66 grams of nitrogen. From experiments on starving normal animals we know that the elimination of a similar amount of nitrogen per day would have kept up had no phlorhizin been given. The administration of phlorhizin resulted in the immediate appearance of glycosuria and in a concomitant rise in the protein metabolism. This took

TABLE II  
*Experiment by Lusk Amer Journ of Physiol, 1, p 397*

PERIOD	WEIGHT	TOTAL NITROOEN	NITROOEN PER HOUR	TOTAL GLUCOSE	OLUCOSE PER HOUR	D N	REMARKS
I	21 4	4 04	0 168				Fasting
II		4 17	0 174				Fasting
III		12 66	0 527	63 55	2 65	5 02	Phlorhizin
IV	20 1	18 76	0 782	65 30	2 72	3 38	Phlorhizin
V		18 57	0 774	65 84	2 74	3 54	Phlorhizin
VI		17 29	0 720	64 80	2 70	3 74	Phlorhizin

TABLE III

Dog 19

DATE	PERIOD	WEIGHT	HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D N	REMARKS
April, 1912 8 { (Third starving day)	I	12 79	4 0	0 388	0 097				Normal and starving Normal and starving Phlorhizin administra- tion com- menced
8	II		16 25	1 83	0 1126				
9	III		3 0	0 455	0 152	4 44	1 48	9 76	
9	IV		3 0	0 490	0 163	4 016	1 335	8 20	
9	V		3 0	0 635	0 2116	3 55	1 183	5 6	
9	VI		3 0	0 777	0 259	3 65	1 22	4 7	
9	VII		12 0	3 82	0 319				
10	VIII	12 41	3 0	1 035	0 345	3 85	1 29	3 72	
10	IX		3 0	1 145	0 382	3 79	1 26	3 32	
10	X		3 0	1 195	0 398	3 77	1 26	3 16	
10	XI		3 0	1 15	0 383	3 80	1 27	3 3	
10	XII		12 0	4 94	0 412	15 18	1 26	3 07	
11	XIII	12 35	3 0	1 25	0 416	4 011	1 337	3 21	

Experiment continued for other purpose

place gradually until it reached its highest level, on the third day, when Lusk's D N ratio became established. If uninfluenced by external factors, it remains at that high level until shortly before death, when it declines slightly.

In table II an experiment is reproduced, which was published by Reilly, Nolan and Lusk<sup>3</sup> about fifteen years ago. Its object here is to illustrate the similarity of the behavior of dogs of different sizes.

In table III a detailed study is presented of the nitrogen and glucose metabolism during the first two days of phlorhizin glycosuria. In this, as in all other phlorhizin experiments, it is to be

<sup>3</sup> Reilly, Nolan and Lusk. *loc cit*

noted that the glucose elimination runs fairly constant throughout the course of the experiment. The nitrogen, however, is a very variable factor, and it is always the variable factor in the D N quotient.

Before we attempt to further discuss the causes of this peculiar rise in the protein metabolism, it will be well to review the theories advanced in explanation of the mechanism of phlorhizin glycosuria.

## II

Von Mering,<sup>9</sup> in his early experiments on phlorhizin glycosuria, found a concentration of 0.09, 0.095 and 0.082 per cent of glucose in the blood of dogs which excreted 6.5, 9.2 and 8.6 per cent of glucose in the urine. He demonstrated that hyperglycaemia was not a necessary factor in the production of glycosuria, and suggested that the kidney might be the seat of the changes brought about by the phlorhizin. Minkowski<sup>10</sup> corroborated the findings of Von Mering and also showed that after the administration of phlorhizin, there was a decided decrease in the sugar concentration of the blood, and that nephrectomy was not followed by an increase in the glucose concentration. These findings practically paved the way for the classical experiments of Zuntz.<sup>11</sup> He injected some phlorhizin in the renal artery of one side and was able to demonstrate the appearance of glycosuria on that side some time before it appeared on the other. It took fully half an hour before the quantity of glucose eliminated on the second side equalled that of the first. This work was further strengthened by the results of Biedl and Kolisch,<sup>12</sup> in which they demonstrated the fact that the perfused extirpated kidney becomes permeable to sugar after the addition of phlorhizin.

If the kidneys were not the only seat of attack of the phlorhizin, and if phlorhizin *per se* had any influence on the sugar burning capacity of the cells of the body, nephrectomy should have been followed by a rise in the glucose concentration of the blood, as is

<sup>9</sup> Von Mering *Zeitschr f klin Med*, **xvi**, p. 433, 1889.

<sup>10</sup> Minkowski *loc cit*.

<sup>11</sup> Zuntz *Du Bois-Reymond's Archiv f Physiol*, 1895, p. 570.

<sup>12</sup> Biedl and Kolisch *Verhand d 18ten Kongresses f innere Medizin*, p. 573, 1900.

the case in pancreatic diabetes (Minkowski, *et al*) This, however, did not take place

O Loewi,<sup>13</sup> employing a very clever method of experimentation, arrived at the conclusion that in normal animals the glucose does not exist in the blood in a free crystalloidal form, but combined with one of the colloids This colloidal combination cannot pass the kidney membrane The phlorhizin affects the cells of the tubules of the kidneys in such a way as to bring about a dissociation of the glucose from the colloids The glucose is then secreted into the tubules There, by its power of adsorption, it holds on, and thus prevents the reabsorption by the tubules of the water which has been filtered through the glomeruli, resulting in a "passive" diuresis

Loewi's conclusions do not stand unchallenged Michaelis and Rona,<sup>14</sup> and Rosenfeld and Ascher<sup>15</sup> do not believe that the sugar circulates in the blood in a combined state However this may be, as far as our present means of investigation go, the fact stands proven that the kidneys are the only or at least the most important seat of attack of the phlorhizin The character of the attack, however, is still in the domain of speculation

Normally, when sugar is fed to an animal, there is an increase in the concentration of the glucose in the blood There is an increase in the process of diffusion of glucose from the blood to the cells of the body, where it is either oxidized or converted into the still more stable form of glycogen, to await further need The concentration in the blood then comes down to its normal level

In starvation, the process is reversed The stream of diffusion is in the opposite direction to maintain the concentration of glucose in the blood, which undoubtedly always tends to sink, because of the withdrawal of glucose from the blood by the very active organs which themselves do not store glycogen to any considerable extent, like the heart and glands

From the aforesaid, one can easily conceive that some sort of equilibrium normally exists between the amount of glycogen in the

<sup>13</sup> O Loewi *Arch f exp Pathol u Pharm*, xlviii, p 410 1902, 1 p 326, 1903

<sup>14</sup> Michaelis and Rona *Biochem Zeitschr*, vii, p 329, 1907

<sup>15</sup> Rosenfeld and Ascher *Centralbl f Physiol*, vii, No 14, 1905



cells and the concentration of sugar in the blood After the administration of phlorhizin, when sugar begins to be poured out by the kidneys, this equilibrium is disturbed, with the negative balance on the side of the blood From the fact that the blood shows a remarkable persistence in maintaining its glucose concentration at the level of about 0.07 to 0.10 per cent in different conditions of nutrition in health and disease, except in pancreatic diabetes and diabetes mellitus, it becomes evident that there must be some teleological reason for it And that these factors should be at play in mobilizing the carbohydrates from the glycogen depots in cases where the blood is depleted of its glucose (phlorhizin glycosuria) seems very probable

With the rapid withdrawal of glucose from the blood by the kidneys, the concentration of the glucose in the blood tends to sink The diminution, however, is adequately compensated for as long as there is plenty of glycogen *Should the supply of the latter become low, the protein will be called upon to contribute to the supply of glucose in the blood*

The protein metabolism on the first day of phlorhizin glycosuria is always increased, but the increase depends upon the amount of glycogen the animal has at its disposal If the animal has been in good condition of nutrition with an abundant supply of glycogen, the depletion of the system of its glucose is compensated for almost entirely by the glycogen This is evident from the experiment recorded in table III, in which the D/N ratio is exceedingly high during the first six hours of the glycosuria, while there is but a slight rise in the nitrogen elimination The protein reaches the highest level of catabolism at about the second or third day of the glycosuria, when it is the sole contributor to the glucose supply of the blood At this stage, Lusk's quotient of  $D/N = 3.61$  becomes established It represents the maximum destruction (Einschmelzung) of protein in its attempt to maintain the glucose concentration of the blood, and it also represents the maximum amount of glucose that can be produced from protein

In the foregoing, the hypothesis has been presented that the diminution in the concentration of glucose in the blood is an essential factor in the rise of protein metabolism Experimental evidence which leads to that conclusion will now be presented

## III

The sparing influence of carbohydrates on the protein metabolism has been well established. Lusk's<sup>16</sup> early researches show to what extent the protein metabolism will be increased after suddenly removing carbohydrates from the diet, whereas Rubner,<sup>17</sup> Lander-gren,<sup>18</sup> Cathcart<sup>19</sup> and others have shown it to have the power of reducing the starvation requirements of protein.

Kayser<sup>20</sup> showed that by replacing the carbohydrates from the diet by an isodynamic quantity of fat, the nitrogen metabolism increases. Throughout the whole experiment, lasting ten days, the calorific value of the diet was kept fairly high and constant (2556 to 2607 Calories). The amount of nitrogen ingested was 21.1 to 21.5 grams. The nitrogen-free substances of the first four days and of the last three days consisted of 71 grams of fat and 338 grams of carbohydrate. During the three days of the intermediary period, the same amount of nitrogen was ingested, but all of the carbohydrate was replaced by an isodynamic quantity of fat, i.e., 21 grams of nitrogen + 220 grams of fat. The urinary analyses showed the following amounts of nitrogen per day.

		gms	gms	gms	gms
First period of 4 days	Mixed diet	17.4	18.8	19.31	20.1
Second period of 3 days	Protein and fat	22.2	22.9	25.4	
Third period of 3 days	Mixed diet	20.8	18.4	18.8	

Landergren,<sup>21</sup> in a series of very beautiful experiments on man, showed that in specific nitrogen hunger and on a rich carbohydrate diet, the nitrogen metabolism will be reduced to a minimum and will reach the lowest level on the fourth day, when less than 4 grams will be eliminated. These findings have since been corroborated by Cathcart.<sup>22</sup>

In experiment VIII of his researches, Landergren gave a man, weighing 69.7 kilos, a diet of 0.2 gram of nitrogen, 737.5 grams of

<sup>16</sup> Lusk *Zeitschr f Biol*, LVII, p. 459, 1890.

<sup>17</sup> Rubner *Gesetze des Energieverbrauches bei der Ernährung*, pp. 71, 79.

<sup>18</sup> Landergren *Skand Arch f Physiol*, XIV, p. 112, 1903.

<sup>19</sup> Cathcart *Journ of Physiol*, XXIV, p. 311, 1909.

<sup>20</sup> Kayser *Beiträge zur Lehre vom Stoffwechsel*, II, p. 1, 1894.

<sup>21</sup> *Loc cit*.

<sup>22</sup> *Loc cit*.

carbohydrate and 17 grams of alcohol per day for four days. The calorific value of this diet was 3150 Calories or 45.2 Calories per kilo of body weight. On the fifth day all of the carbohydrates of the diet were replaced by an isodynamic quantity of fat (304 grams fat, 30.4 grams alcohol = 3048 Calories). This diet was given for three days. The urinary analyses showed the following amounts of nitrogen per day

	Carbohydrate period				Fat period		
0	I	II	III	IV	V	VI	VII
12.76	8.91	5.15	4.30	3.76	4.28	8.86	9.64

In his paper on the Influence of Carbohydrates and Fats on Protein Metabolism, Cathcart<sup>23</sup> reports a similar experiment performed on a man in specific nitrogen hunger. He was kept for five days on a diet containing 454 grams of banana meal and 230 grams of honey (32 Calories per kilo of body weight). On the sixth and seventh days the carbohydrate was replaced by a diet of butter and cream, a little higher than isodynamic (35 Calories per kilo). The urinary analyses showed the following amounts of nitrogen per day

Carbohydrate period					Fat period	
I	II	III	IV	V	VI	VII
6.79	6.40	4.77	4.79	4.39	4.83	8.13

These two experiments stand in absolute agreement in showing that the catabolism of protein in the body on a pure carbohydrate diet is at least one-half as much as on a diet containing an isodynamic quantity of fat.

How can we explain this peculiar difference between two foodstuffs, which dynamogenetically replace each other quantitatively?

The total metabolism of the cells of the body, according to Rubner<sup>24</sup> is determined by its requirement for kinetic energy. It does not make much difference which of the foodstuffs is called upon to satisfy this requirement. It probably depends upon the concentration of the different foodstuffs in the fluids of the body including those of the protoplasm. Here, the protein, carbohydrates and fats replace each other in isodynamic quantities. Assuming

<sup>23</sup> *Loc cit*

<sup>24</sup> Rubner v. Leyden's *Handbuch*, 1, p. 78, 1893

that the cells exercise free selection and that the concentration of the body fluids is equally rich in all of the foodstuffs, the protein will be attacked first, carbohydrates next and lastly the fats. According to E Voit<sup>25</sup> this order is determined by the affinity of the cells for the individual foodstuffs. In addition, the carbohydrates, because of their aldehyde and ketone groups, probably maintain a much more labile equilibrium than do the fats, and so burn much quicker, and thus spare the burning of protein. Rubner also believes that the relative sizes of the molecules of carbohydrates and fats have a great deal to do with the velocity of absorption and penetration into the cells, and that these factors give the advantage to the carbohydrates.

Landergren, however, does not accept this hypothesis. From his own experiments, and those of Talquist,<sup>26</sup> he concludes that the physical and chemical differences between the carbohydrates and fats are not sufficient to explain the difference in their protein sparing properties. He believes that when a condition arises in which the animal body has no carbohydrate at its disposal the body must prepare it from its own material. Since a physiological transformation of fat into sugar has never been proven, he assumes that the protein is the sole contributor to the formation of glucose. The constancy of the concentration of sugar in the blood in cases of prolonged starvation speaks very much in favor of this hypothesis. The giving of carbohydrate therefore spares that amount of protein catabolism, while a diet of fat does not. The protein metabolism must then be assumed to consist of three component parts.

1 The "minimal nitrogen requirement" which represents the amount of protein that is catabolized during specific nitrogen hunger, when the body receives all its calorific requirements in the form of carbohydrates, or carbohydrates and fats in equal calorific quantities. This amounts to about 4 grams of nitrogen per day in a full grown man, and corresponds to Rubner's "wear and tear quota."

2 The "dextrose nitrogen" which represents the amount of protein that is catabolized in excess of the "minimal nitrogen

<sup>25</sup> E Voit and A Korkunoff *Zeitschr f Biol*, LXXII, p 135, 1895

<sup>26</sup> Talquist cited by Landergren

requirement" in case of specific nitrogen hunger and in the absence of carbohydrates from the diet, *i e*, when the calorific requirement is covered exclusively by fat. This amounts to about 4 or 5 grams of nitrogen per day.

3 The "complementary nitrogen" which represents the amount of protein catabolized for dynamogenetic purposes in cases of starvation in excess of 1 and 2, or when large quantities of protein are added to the diet.

The nitrogen value of 1 cannot be reduced by carbohydrates or fats. It can be *replaced* only by protein. The nitrogen of 2 can be *spared* by a sufficient supply of carbohydrates (50 per cent of calorific requirements). It cannot be spared by fat. The nitrogen of 3 can be spared by any foodstuff.

Phlorhizin glycosuria and pancreatic diabetes offer a very good opportunity for the study of this problem. In both do we meet with a tremendous rise in the protein metabolism and in both is there an absence of glucose combustion. Thanks to the researches of Lusk, the relationship between the protein metabolism in phlorhizin glycosuria and normal starvation has been worked out to a high degree of accuracy. A perusal of the literature on pancreatic diabetes, however, reveals the fact that in very few experiments has any attention been paid to this. The works of Falta, Grote and Stähelin<sup>27</sup> cannot be relied upon because of the fact that their animals had febrile temperature, which is a very disturbing factor in the study of protein metabolism.

The only satisfactory experiments are those of Falta and Whitney,<sup>28</sup> part of which is reproduced in table IV. For four days they studied the protein metabolism of a normal starving dog. On the fifth day the pancreas was removed. The analysis of the urine commenced two hours after the extirpation of the pancreas.

The average daily elimination of nitrogen during the foreperiod was  $\frac{5.35 + 3.39 + 3.86 + 4.21}{4} = 4.20$  grams. The highest

amount of nitrogen eliminated during the diabetes was 12.01 grams, *i e*, an increase of 286 per cent above the starvation requirements. This is decidedly lower than the increase in the protein catabolism.

<sup>27</sup> Falta, Grote and Stähelin. *Loc cit*.

<sup>28</sup> Falta and Whitney. *Beitr z chem Physiol u Pathol*, vi, p 224, 1908.

TABLE IV

PERIOD	WEIGHT	NITROOEN	NITROOEN PER HOUR	GLUCOSE	D N	REMARKS
I	17 35	5 35				Normal starv ing
II	17 17	3 39				
III	16 88	3 86				
IV	16 53	4 21				

*Extirpation of pancreas*

V*	15 68	11 52	0 48	27 02	2 35	
VI	14 89	11 83	0 49	38 04	3 15	
VII		11 98	0 50	34 54	2 88	
VIII	14 08	12 01	0 50	38 25	3 18	

Analyses were made for fourteen hours calculated by the author for twenty four hours

that is usually associated with phlorhizin glycosuria. There is no question but that the increase in nitrogen elimination in pancreatic diabetes is due to dynamogenetic reasons only, for this condition is always associated with hyperglycaemia, and, under ordinary circumstances,<sup>29</sup> with complete cessation of the utilization of glucose.

In phlorhizin glycosuria, under conditions of not too great administration of carbohydrates, we also meet with complete cessation of the utilization of glucose, but this condition is associated with a decided hypoglycaemia and a rise in the protein metabolism above the starvation requirements, to a much greater extent than in pancreas diabetes. If Landergren's hypothesis is true, that the protein metabolism is to a certain extent dependent upon the concentration of glucose in the blood and body fluids, then, the feeding of glucose to phlorhizinized dogs, in quantities small enough not to exceed "the diffusion level," ought to be followed by a lowering in the protein metabolism (*i e*, sparing of fraction 2. See page 439). On the other hand, the giving of a similar quantity of glucose to a depancreatized animal ought to be followed by no depression in the protein metabolism.

The experiments reported below were performed with the object of testing the truth of this hypothesis.

<sup>29</sup> That is, if not accompanied by the administration of too large quantities of carbohydrate.

TABLE V  
Dog 11 Twenty-four-hour periods

PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL OLUCOSE	D N	REMARKS
III	17 53	14 40	52 08	3 62	Fasting
IV	17 24	9 32	103 10	11 06	75 grams of glucose given per os in six doses
V	16 86	14 00	50 95	3 64	150 grams of glucose as above
VI	16 60	7 18	127 17	17 71	
VII	16 25	7 78	56 29	7 23	

TABLE VI  
Dog 15 Twenty-four-hour periods

II	12 25	12 43	44 67	3 59	100 grams beef heart and 75 grams lard.
III	12 00	10 50	36 93	3 51	
IV	11 34	9 08	59 78	6 58	
V	11 34	9 08	59 78	6 58	25 grams glucose given per os during day
VI	11 21	10 75	40 39	3 75	100 grams beef heart and 75 grams lard

TABLE VII  
Dog 17 Twelve-hour periods

PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL OLUCOSE	D N	REMARKS
I		5 32	20 19	3 79	Fasting
II	10 31	4 14	46 78	11 30	49 grams of glucose given per os
III		4 35	27 30	6 28	
IV	9 93	5 00	20 49	4 10	

TABLE VIII  
Pancreas diabetes Dog 12 Twenty-four hour periods

I	7 93	7 09	16 92	2 38	40 grams of glucose given per os
II	7 68	6 48	17 57	2 71	
III		6 88	64 80	9 42	
IV	7 08	12 41	39 54	3 19	300 grams of beef heart
V		14 25	36 27	2 54	400 grams of beef heart
VI	7 07	15 46	40 07	2 60	

Dog 11 was phlorhizinized in the usual manner and the D N ratio established. Seventy-five grams of glucose dissolved in water and divided into six doses were given *per os* on the fourth day of the glycosuria. One hundred and fifty grams were given on the sixth day.

From the D N ratio on the third and fifth days, we may safely assume that the phlorhizin intoxication was complete and that the protein metabolized on the fourth, sixth and seventh days yielded 3.6 grams of glucose for every gram of nitrogen.

The amount of glucose eliminated on the fourth day was 103.1 grams. By subtracting 33.55 grams, which originated from the protein ( $9.32 \times 3.6$ ), we find that 69.5 grams of the 75 grams of glucose fed were eliminated unburnt. By applying similar calculations to the results obtained on the sixth and seventh days, we find that the protein metabolized during the sixth day yielded ( $7.18 \times 3.6$ ) = 23.85 grams of glucose and during the seventh day ( $7.78 \times 3.6$ ) = 28.01 grams. The total amount of glucose eliminated during these two days was 183.46 grams. By subtracting the glucose that was derived from the protein, we find that 131.6 grams of the 150 grams of glucose ingested were eliminated unburnt.

The nitrogen metabolism was diminished by a little more than 5 grams on the fourth day and was reduced almost 50 per cent on the sixth and seventh days. If the increase in the protein metabolism in phlorhizin diabetes were due to dynamogenetic reasons only, the burning of 5.5 grams of glucose on the fourth day could not have spared the combustion of 31.8 grams of protein. Nor could the burning of 18.4 grams of glucose on the sixth and seventh days have spared as much as 81 grams of protein.

It is also noteworthy in this experiment that the 150 grams of glucose given within twelve hours were not eliminated completely during the first twenty-four hours, but were carried over to a great extent to the second twenty-four hours.

Dog 15 was phlorhizinized and fed 75 grams of lard and 100 grams of beef heart per day. This covered approximately his calorific requirements. During period V, 25 grams of glucose were added to his diet and were administered in six doses. The amount of glucose eliminated on that day was 59.78 grams. By subtracting the amount of glucose that originated from the 9.08 grams



of protein  $[59.78 - (9.08 \times 3.6) = 27.1]$  we find that 27.1 grams of extra glucose were eliminated on that day. It is absolutely certain that none of the glucose fed was burnt, and still there is a decided diminution in the protein metabolism.

Dog 17 was treated like Dog 11. During period II the animal received *per os* 49 grams of glucose. The D/N ratio in period IV was still high, showing that extra glucose was still being eliminated. The total amount of glucose eliminated during periods II, III and IV was 94.57 grams. The total nitrogen eliminated during these periods was 13.49 grams, which could give rise to 48.56 grams of glucose. By subtracting this from the total, we find that from the 49 grams of glucose administered, 46 were recovered in the urine. The total nitrogen, in this case as in the two previous experiments, was considerably reduced during the "glucose" period.

Dog 12 had its pancreas removed about three weeks before the experiment was commenced. The operation was performed by Dr. J. E. Sweet of the Department of Experimental Surgery. That the pancreas was completely removed, we assume from the character of the D/N ratio, which was close to the one established by Minkowski. On the third day of the experiment the animal received 40 grams of glucose, administered *per os* in six doses.

This experiment shows, in a very striking manner, the difference between pancreatic and phlorhizin glycosuria. *Whereas the giving of glucose to phlorhizinized animals is invariably associated with a lowering of the protein metabolism, this phenomenon is not observed in pancreatic diabetes.*

These experiments support the hypothesis of Landergren. They show that *a certain amount of protein in phlorhizin glycosuria can be spared by glucose although the glucose is not burnt, but is eliminated quantitatively in the urine.*

The question of dynamogenesis can be left out entirely, for in Dog 15 every gram of glucose was recovered in the urine. We must then assume the existence of a fraction of protein metabolism that is spared by the mere presence of glucose in the circulation. It exists as a factor distinct from the "wear and tear quota" (minimal nitrogen requirement) and also distinct from the "complementary fraction."

One other important fact to be noted is the difference between the nitrogen curves presented by the phlorhizinized and depancrea-

tized dogs during the onset of glycosuria. In phlorhizinized animals the nitrogen reaches the highest level on the second or third day, whereas in the depancreatized dog, the protein metabolism rises at once to the highest level (see tables I, II, III and compare with IV)

#### SUMMARY

1 Experiments were performed which showed that in phlorhizin glycosuria the protein metabolism rises in part because of the hypoglycaemia that is present in that condition

2 The giving of small quantities of glucose to phlorhizinized animals results in a sparing of protein, although all of the glucose is eliminated in the urine and none of it is burnt. This lends support to Landergren's hypothesis, which assumes that in starvation, a certain fraction of protein is metabolized for the formation of glucose, and that this fraction can be spared by carbohydrates and not by fat

3 The protein metabolism above the starvation requirement in pancreatic diabetes does not rise as high as in phlorhizin diabetes

4 The giving of glucose to a dog with pancreatic diabetes does not spare any protein

5 It is suggested that the protein metabolism is lower in pancreatic than in phlorhizin diabetes because of the hyperglycaemia which prevents the catabolism of the so-called "dextrose protein"



# CONCERNING THE ORGANIC PHOSPHORIC ACID COMPOUND OF WHEAT BRAN

PRELIMINARY REPORT    THIRD PAPER ON PHYTIN

BY R. J. ANDERSON

(From the Chemical Laboratory of the New York Agricultural Experiment  
Station, Geneva, N. Y.)

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## INTRODUCTION

In the last two papers dealing with the chemistry of phytin<sup>1</sup> various salts of phytic acid with inorganic bases, as well as various phosphoric and pyrophosphoric acid esters of inositol, have been described. In connection with this work the subject of the organic phosphorus compound of wheat bran was taken up.

Patten and Hart<sup>2</sup> had shown that wheat bran contains an organic phosphorus body which on cleavage with 30 per cent sulphuric acids in a sealed tube gave inositol as one of the products of decomposition. They also obtained an acid from a 0.2 per cent hydrochloric acid extract of bran which on analysis gave results corresponding very closely with the theoretical composition of phytic acid, or, as the substance was then called, "anhydro-oxymethylene di-phosphoric acid." These results led them to believe that the substance which they had isolated was identical with the organic phosphorus compound described by Palladin,<sup>3</sup> Schulze and Winterstein<sup>4</sup> and later by Winterstein<sup>5</sup> and which was finally obtained in pure form by Posternak<sup>6</sup> who gave the substance the name "phytin."

<sup>1</sup> This *Journal*, vi, p. 471, vii, p. 97, Technical Bull. 19 and 21, N. Y. Agric. Exp. Station, 1912.

<sup>2</sup> *Amer. Chem. Journ.*, xxxi, p. 566, 1904.

<sup>3</sup> *Zeitschr. f. Biol.*, p. 199, 1894.

<sup>4</sup> *Zeitschr. f. physiol. Chem.*, xxi, p. 90.

<sup>5</sup> *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 2299.

<sup>6</sup> *Rev. gén. bot.*, vii, pp. 5, 65, 1900, *Compt. rend. acad. d. sci.*, cxxxvii, pp. 202, 337, 439, 1903.

## 448      Organic Phosphoric Acid of Wheat Bran

This substance, isolated by Patten and Hart and assumed by them to be phytin, has since been regarded as such by Suzuki and Yoshimura,<sup>7</sup> Neuberg,<sup>8</sup> Forbes<sup>9</sup> and others. These authors do not, however, report any complete analyses of the substance.

Since the investigation of the organic phosphorus compound of wheat bran by Patten and Hart, several feeding experiments, some of which have not yet been published, to determine the physiological effect of phytin have been carried out at this institution by Dr. Jordan. In these experiments it has been found that the effect of pure phytin salts differs decidedly from that obtained by feeding varying quantities of washed and unwashed wheat bran.<sup>10</sup> These anomalous results could not be explained on the assumption that only "phytin" was removed from the bran by washing. The problem was still more complicated by the fact that previous work had shown that very little besides phosphorus compound and inorganic bases had been removed from the wheat bran in the process of washing or leaching.<sup>11</sup>

In the hope of throwing some light on this subject, the chemical investigation of the products removed from wheat bran by washing it in dilute acid was again taken up. The chief object was to isolate and identify the organic phosphorus body and to determine what bases were associated with it.

If ordinary wheat bran be extracted with 0.2 per cent hydrochloric acid and the resulting filtered extract precipitated with alcohol, a body is obtained which, after repeated precipitations from 0.2 per cent hydrochloric acid with alcohol, shows a relatively uniform composition. The composition varies somewhat depending upon the conditions under which the substance is prepared, but on an average it has been found to be about as follows: C, 21.0, H, 3.5, P, 14.0 per cent. The substance also contains calcium, magnesium, potassium and sodium in varying amounts, together with traces of iron, and it always contains nitrogen varying from 2.1 to 0.4 per cent. The nitrogen, however, is not present as ammonia. The compound reduces Fehling's solution on boil-

<sup>7</sup> *Bull. Coll. of Agric. Tokyo*, vii, p. 498.

<sup>8</sup> *Biochem. Zeitschr.*, xvi, p. 405.

<sup>9</sup> Bull. 215, Ohio Agric. Exp. Station.

<sup>10</sup> *Amer. Journ. of Physiol.*, xvi, p. 268, 1906.

<sup>11</sup> *Ibid.*, xvi, pp. 274, 304, 1906.

ing It gives reactions with oreine and phloroglucine and yields furfurol when distilled with 12 per cent hydrochloric acid It was first described by Patten and Hart<sup>12</sup> although they do not mention the above reactions As previously stated they considered it identical with phytin although according to their analysis it had the following composition

C, 18.52, H, 3.83, P, 16.38, Ca, 1.13 Mg, 5.80, K, 2.60, N, 0.37 per cent

It will be noticed at once that for a salt of phytic acid the above compound has about 10 per cent *too much carbon* and about 6 per cent *too little phosphorus*

After isolating and purifying some of the substance, as will be described in the experimental part, a product was finally obtained which had the composition first mentioned above It was believed at first that it was an impure phytin compound, probably associated with some carbohydrate group and some complex nitrogen-containing body All attempts to prepare any of the characteristic salts of phytic acid from the substance have failed We have found, however, that it is possible by the action of barium hydroxide to separate the substance into two constituents, one of which is an organic phosphoric acid free from nitrogen, the other a nitrogenous substance also containing phosphorus in organic combination The latter has, however, not been obtained in pure form It has not been analyzed and its nature is at present entirely unknown

The barium salt of the nitrogen-free body corresponds to the formula  $C_{25}H_{55}O_{54}P_9Ba_5$  The isolation of the free acid,  $C_{25}H_{55}O_{54}P_9$ , corresponding to the above barium salt, has not succeeded Attempts to isolate it led to an organic phosphoric acid, lower in carbon and higher in phosphorus, of the composition  $C_{20}H_{45}O_{49}P_9$  In the process of isolation apparently the elements of one pentose are split off



If, in the preparation of the above barium salt from the crude substance, the solution is allowed to stand in contact with dilute hydrochloric acid for any length of time a barium salt is obtained corresponding to the second acid,  $C_{20}H_{45}O_{49}P_9Ba_5$ , from which salt the free acid may be generated The barium salt,  $C_{25}H_{55}O_{54}P_9Ba_5$ ,

<sup>1</sup> *Loc cit*

yields furfural on distillation with 12 per cent hydrochloric acid but the salt,  $C_{20}H_{45}O_{49}P_9Ba_5$ , does not do so

The acid,  $C_{20}H_{55}O_{49}P_9$ , apparently represents the nucleus of the molecule of the organic phosphorus compound, as it has been found impossible to obtain any simpler organic phosphoric acid from it by treatment with acids. On boiling with normal sulphuric acid at ordinary pressure it is slowly decomposed with formation of phosphoric acid and *reducing bodies*, apparently carbohydrates, as the solution reduces Fehling's solution and gives reactions with orcin and phloroglucine, but no trace of inositol could be isolated. The unchanged portion isolated from the reaction mixture has exactly the same composition as it had before boiling, which indicates that the molecule is gradually broken up into reducing bodies and phosphoric acid without suffering any intermediate or partial decomposition. On heating the substance in a sealed tube with 5 N sulphuric acid the cleavage appears to go in another direction, for in this case 90 per cent of the total carbon was recovered in the form of inositol and absolutely no reducing bodies were present in the reaction mixture. No explanation can be offered at this time concerning this peculiar behavior towards sulphuric acid under ordinary pressure and in a sealed tube.

It is evident that this compound is *not phytin*. The only similarity between these substances is found in that they are both organic phosphoric acids and that when heated in a sealed tube with acids they yield inositol as one of the products of decomposition. Whether this new compound contains the inositol as such or whether it is only formed in the process of decomposition cannot be definitely determined at this time. However, if it were a complex compound of inositol and phosphoric or pyrophosphoric acid, the isolation of inositol should be possible after cleavage with dilute acid at ordinary pressure. As has been stated this cannot be done and, moreover, the empirical formula of the substance can hardly be brought into accord with any inositol compound.

The substance is probably similar to, if not identical with, the glucophosphoric acid described by Levene<sup>13</sup>. The same author<sup>14</sup>

<sup>13</sup> *Journ Amer Chem Soc*, xiv, p 190, 1902, *Amer Journ of Physiol*, vii, p 11, 1903

<sup>14</sup> *Biochem Zeitschr*, xvi, p 399

also described an organic phosphoric acid compound isolated from hemp seed which gave reactions for pentose or glucuronic acid. Since phytin does not give these reactions, as stated by Neuberg,<sup>15</sup> it is very probable that the products examined by Levene were of the same nature as that described in this paper.

The chief support of the assumption of Patten and Hart<sup>16</sup> that the organic phosphorus compound of wheat bran was phytin was no doubt based upon the fact that they had obtained a substance from dilute hydrochloric acid extract of bran which corresponded closely in composition with that required for the "anhydro-oxyethylene di-phosphoric acid" of Posternak. Serious objection, however, must be raised against their method of isolating this substance in that they made absolutely no effort to remove *inorganic phosphates*. From the work of Hart and Andrews<sup>17</sup> they believed themselves justified in considering the inorganic soluble phosphates present in wheat bran as a negligible quantity. While we cannot enter into any discussion of the above work here, it is to be noted that in the determination of the soluble inorganic phosphates in plant constituents Hart and Andrews extracted the material with 0.2 per cent hydrochloric acid for fifteen minutes and determined the inorganic phosphorus in the filtered extract by precipitating with nearly neutral ammonium molybdate. By this method they found 0.036 per cent inorganic phosphorus in wheat bran. The total amount of phosphorus compounds soluble in 0.2 per cent hydrochloric acid was found to be equivalent to 0.951 per cent phosphorus. The inorganic phosphorus found by the above authors is therefore equal to 3.78 per cent of the total phosphorus soluble in 0.2 per cent hydrochloric acid.

Suzuki and Yoshimura<sup>18</sup> report phosphorus determinations in wheat bran. They found 0.638 per cent of phosphorus soluble in 0.2 per cent hydrochloric acid, of which 0.050 per cent was inorganic and 0.579 per cent organic. The inorganic phosphorus found in this case is therefore equal to 8.63 per cent of the total phosphorus soluble in 0.2 per cent hydrochloric acid.

<sup>15</sup> *Biochem Zeitschr*, xvi, p 405

<sup>16</sup> *Loc cit*

<sup>17</sup> Bull 238 N Y Agric Exp Station, 1903

<sup>18</sup> *Bull Coll of Agric, Tokyo*, vii, p 498



The work of Forbes and associates<sup>19</sup> seems to show that the time allowed by Hart and Andrews, fifteen minutes, is not sufficient for complete extraction and that neutral molybdate solution is not suitable for precipitation in all cases, that when three hours is allowed for extraction a considerably larger amount of inorganic phosphorus is obtained

In the preparation of the "phytin" products from wheat bran Patten and Hart<sup>20</sup> do not mention any definite time allowed for extraction but only state that "the bran was extracted for several hours with 0.2 per cent hydrochloric acid," apparently therefore a longer time than allowed by Hart and Andrews in their determinations of inorganic phosphorus

When wheat bran is digested for several hours in 0.2 per cent hydrochloric acid we have found that the resulting extract always contains a considerable quantity of inorganic phosphates. Quantitative determinations have, however, not been carried out and we are at present unable to state whether the inorganic phosphates were present in the bran originally or if they have been formed by hydrolysis of the organic phosphorus compounds, but we purpose to take up this phase of the subject later

On precipitating a bran extract, prepared as indicated above, with any of the usual reagents for the isolation of the organic phosphorus compound the inorganic phosphates are more or less completely precipitated at the same time. In order to remove these inorganic phosphates we have found it necessary to repeatedly precipitate the substance from 0.2 or 0.5 per cent hydrochloric acid with alcohol. In other words the substance has been reprecipitated until the dilute nitric acid solution of the resulting product does not give any immediate reaction with ammonium molybdate. The slight amount of phosphomolybdate precipitated from the solution on longer standing is no doubt due to cleavage rather than to admixed inorganic phosphates

In order to determine if any barium salt of different composition from those discussed above could be prepared directly from wheat bran extract, a 0.2 per cent hydrochloric acid extract of bran, prepared as before, was precipitated with barium chloride and alcohol. The substance was purified by precipitating from 0.5

<sup>19</sup> *Loc cit*

<sup>20</sup> *Loc cit*

per cent hydrochloric acid solution with alcohol until it gave no immediate reaction with ammonium molybdate (cf experimental part) Analysis showed that this compound contained a higher percentage of carbon and lower phosphorus than the barium salt prepared from the previously isolated crude substance and it gave a larger amount of furfural on distillation with 12 per cent hydrochloric acid By treating this compound with dilute sulphuric acid for a short time some reducing body was split off and the organic phosphorus substance finally isolated from the reaction mixture corresponded in composition with the barium salt first prepared, viz,  $C_{25}H_{55}O_{54}P_9Ba_5$  This compound is, however, easily transformed into  $C_{20}H_{55}O_{49}P_9$  as has already been shown

Since we have been unable to isolate any compound from wheat bran corresponding in composition to a salt of phytic acid we have come to the conclusion that wheat bran *does not contain phytin* and that the compound  $C_{20}H_{55}O_{49}P_9$  is the only organic phosphoric acid existing in bran It appears, however, that in its natural condition in the bran one or more as yet unidentified reducing bodies, which yield furfural on distillation with hydrochloric acid and which are easily split off by the action of dilute acids, are loosely bound to this nucleus

The so-called "anhydro-oxyethylene di-phosphoric acid" analyzed by Patten and Hart was undoubtedly a mixture of the above compound and free phosphoric acid This seems the more probable as they had not made any effort to remove inorganic phosphates in the preparation of their acid

The empirical formulas suggested in this paper are of course purely tentative We are now preparing larger quantities of the substance from wheat bran and hope shortly to be able to report further concerning this compound Various other cereals and feeding stuffs are also being examined to determine whether they contain phytin or if this other organic phosphoric acid compound is present

#### EXPERIMENTAL PART

##### *Preparation of the organic phosphorus compound from wheat bran*

The bran was digested with 0.2 per cent hydrochloric acid overnight and the extract after straining and filtering was precipitated

with alcohol according to the method of Patten and Hart (*loc cit*) The resulting precipitate was purified by precipitating five times from 0.2 per cent hydrochloric acid with alcohol. From 500 grams of bran 2.5 grams of substance were obtained as a white amorphous non-hygroscopic powder. It is readily soluble in its own weight of water, forming a thick light amber colored solution of pleasant and characteristic but faint acid odor. The substance reduces Fehling's solution on boiling and it gives the orcin and phloroglucine reactions. The aqueous solution is acid to litmus paper. It is precipitated by alkalis and solutions of salts of other metals. Warmed with dilute nitric acid and ammonium molybdate it does not give any immediate precipitate but on standing for several hours a trace of yellow phosphomolybdate is precipitated.

After drying at 120° the substance was analyzed

0.1642 gram substance gave 0.0522 gram  $H_2O$  and 0.1285 gram  $CO_2$

0.0360 gram substance gave 0.0428 gram  $Mg_2P_2O_7$  for P

0.1720 gram substance gave 0.0264 gram  $Mg_2P_2O_7$  for Mg

0.1720 gram substance gave 0.0054 gram CaO

0.3897 gram substance gave 0.0085 gram N (Kjeldahl)

The substance contained only a very small quantity of K

Found C, 21.34, H, 3.55, P, 13.87, Ca, 2.24, Mg, 3.35, N, 2.18 per cent

A larger quantity of the product was then prepared by extracting 3 kgs. of bran. After isolating and purifying in the same way as before 47 grams of substance were obtained or about 1.5 per cent of the weight of the bran used. In appearance and properties it was identical with the foregoing.

After drying at 105° in vacuum over phosphorus pentoxide the substance was analyzed

0.2444 gram substance gave 0.0710 gram  $H_2O$  and 0.1940 gram  $CO_2$

0.3936 gram substance gave 0.0149 gram CaO and 0.0361 gram  $Mg_2P_2O_7$

0.3936 gram substance gave 0.0623 gram  $K_2PtCl_6$

Phosphorus and nitrogen determinations were not made

Found C, 21.64, H, 3.25, Ca, 2.70, Mg, 4.77, K, 2.54 per cent

The substance was again precipitated from 0.2 per cent hydrochloric acid, washed and dried as before, when the following results were obtained on analysis

0.1934 gram substance gave 0.0639 gram  $H_2O$  and 0.1523 gram CO

0.3313 gram substance gave 0.0131 gram CaO and 0.0717 gram  $Mg_2P_2O_7$  for Mg

0.3313 gram substance gave 0.0444 gram K PtCl<sub>3</sub>

0.1656 gram substance gave 0.0857 gram Mg P<sub>2</sub>O<sub>7</sub> for P

0.4639 gram substance gave 0.0058 gram N (Kjeldahl)

Found C, 21.47, H, 3.69, P, 14.42, Ca, 2.82, Mg, 4.72, K, 2.15, N, 1.25 per cent

Sodium was not determined but qualitative tests showed that it was present

The reprecipitated substance, 0.3887 gram, distilled with 12 per cent HCl, gave 0.0367 gram phloroglucid

As the composition did not change by reprecipitation it was deemed sufficiently pure to use in the subsequent experiments

It was thought at first that this substance might be phytin mixed with some carbohydrate and some basic nitrogen body. In the hope of separating these and to obtain pure compounds the substance was treated with barium hydroxide and the resulting barium salt purified as follows

#### *Preparation of the barium salt*

Five grams of the substance were dissolved in 10 cc of water and the solution diluted to 200 cc with water. Barium hydroxide was then added until distinctly alkaline and the mixture heated nearly to boiling. It was then filtered hot and washed with hot water, the filtrate being reserved for examination.

The washed barium precipitate was dissolved in just sufficient 0.5 per cent hydrochloric acid, filtered, again precipitated with barium hydroxide, the resulting precipitate dissolved by the careful addition of dilute hydrochloric acid and then precipitated by the addition of a like volume of alcohol. The substance was filtered, washed in dilute alcohol, again dissolved in 0.5 per cent hydrochloric acid and precipitated in the same manner as before. These operations were repeated four times. It was then dissolved in the same strength hydrochloric acid, precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a perfectly white amorphous powder. Yield, 3.9 grams.

The substance was slightly soluble in boiling water. On cooling, however, it does not crystallize out and on concentrating in vacuum it separates in an amorphous form. Alcohol also produces a white amorphous precipitate. Various other methods were tried

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to obtain the substance in crystalline form but without success. On moist litmus paper it shows a strong acid reaction. It was free from nitrogen.

As it was found impossible to crystallize the substance it was analyzed directly after drying at  $130^{\circ}$

0.2564 gram substance gave 0.0604 gram H<sub>2</sub>O and 0.1314 gram CO<sub>2</sub>  
0.2903 gram substance gave 0.1544 gram BaSO<sub>4</sub> and 0.1324 gram Mg P O,  
Found C, 13.97, H, 2.63, P, 12.71, Ba, 31.29 per cent

Of this substance 1.2124 grams were distilled with 12 per cent HCl when 0.0053 gram phloroglucid was obtained.

The composition of the above salt is entirely different from that of a barium phytate. The relation of the numbers found lead to the empirical formula,  $C_{25}H_{55}O_{54}P_9Ba_5 = 2185$ , calculated for this C = 13.73, H = 2.51, P = 12.76, Ba = 31.44 per cent.

### *Examination of the filtrate from the above compound after precipitating with barium hydroxide*

The filtrate was of light amber color. The excess of barium hydroxide was removed with carbon dioxide, filtered and the filtrate concentrated on the water bath, again filtered from traces of barium carbonate and then dried in vacuum over sulphuric acid. There remained a small quantity of a yellowish amber colored, somewhat gummy mass. It contained a large quantity of nitrogen. It did not reduce Fehling's solution and gave only a faint biuret reaction.

The substance is readily soluble in water and is again precipitated by alcohol but it is not precipitated by tannic acid. The aqueous solution acidified with nitric acid gives no reaction with ammonium molybdate.

After combustion the ash was found to contain potassium, sodium and phosphorus. When the crude substance is treated by the Van Slyke method for amino nitrogen a small quantity of nitrogen is liberated.

Lack of time has prevented the further examination of this body and it has not been isolated in pure form.

*Isolation of the free acid from the barium salt*

The barium salt previously described (3.2 grams dry substance) was suspended in 100 cc of water and decomposed with a slight excess of dilute sulphuric acid, the barium sulphate was removed by filtration and the filtrate precipitated with excess of copper acetate. The copper salt was filtered, thoroughly washed in water, suspended in water and decomposed with hydrogen sulphide. The copper sulphide was filtered off and the filtrate concentrated in vacuum to small bulk and finally dried in vacuum over sulphuric acid until it was of a thick syrupy consistency. After drying at 100° to constant weight the substance was analyzed.

0.2907 gram substance gave 0.1052 gram  $H_2O$  and 0.1855 gram  $CO_2$

0.1787 gram substance gave 0.0642 gram  $H_2O$  and 0.1144 gram  $CO_2$

0.1816 gram substance gave 0.1331 gram  $Mg_2P_2O_7$

Found I C, 17.40, H, 4.04, P, 20.43 per cent

II C, 17.46, H, 4.02 per cent

These results lead to the empirical formula,  $C_{20}H_{55}O_{49}P_9$ ,

Calculated for  $C_{20}H_{55}O_{49}P_9$  = 1358 C, 17.67, H, 4.05, P, 20.54 per cent

This compound differs in composition from the barium salt from which it was prepared by  $C_5H_{10}O_5$ , i.e., by the elements of one pentose. This had probably been split off in the decomposition of the barium salt with the dilute sulphuric acid or else by the copper acetate, and if so should be found in the filtrate after the copper salt of the acid had been removed. The filtrate was therefore examined as follows. The copper was removed by hydrogen sulphide and the filtrate, after boiling off excess of  $H_2S$ , was precipitated with excess of barium hydroxide, filtered, and the barium precipitated quantitatively with sulphuric acid and the resulting filtrate evaporated to small bulk in vacuum. The solution was then found to reduce Fehling's solution on boiling and ammoniacal silver nitrate was also reduced. Unfortunately the amount of substance obtained was too small to permit further examination. There is, however, absolutely no doubt that a reducing body, probably pentose, was present.

*Properties of the free acid,  $C_{20}H_{55}O_{49}P_9$* 

Dried in the desiccator it forms first a light amber colored thick syrup which on continued drying forms a thick sticky mass. It is very soluble in water and also readily soluble in alcohol from which

it separates on the addition of ether as a white precipitate which collects on the sides of the test tube in small oily drops

The aqueous solution has a strong acid reaction and a pleasant sharp acid taste and it gives the following reactions

Magnesium acetate does not give a precipitate but the addition of calcium acetate, barium chloride or alcohol causes in this solution a white precipitate

Silver nitrate does not produce any precipitate but the addition of alcohol gives a white amorphous precipitate of the silver salt

It is not precipitated by barium or calcium chlorides but the acetates of these metals and their hydroxides give white amorphous precipitates which are soluble in acetic and mineral acids

Ferric chloride causes a white precipitate which is readily soluble in dilute hydrochloric or nitric acids

The alkali salts are very soluble in water but in these solutions salts of the alkaline earths or the heavy metals produce white precipitates. The addition of alcohol also produces white precipitates

The ordinary molybdate solution does not give any precipitate in dilute solutions of the acid, in concentrated solutions a yellowish white precipitate is obtained. On acidifying with nitric acid and heating, the yellow phosphomolybdate is slowly precipitated

The aqueous solution of the acid is only incompletely precipitated by magnesia mixture. A slight white-colored amorphous precipitate is obtained but the addition of alcohol produces a voluminous white precipitate. This product is however not a pure salt as shown by the following results which were obtained on analysis of the dried precipitate

Found   Mg, 11.29, N, 2.40, P, 16.45 per cent

These numbers do not agree with any formula for a pure magnesium ammonium salt of the above acid

A larger quantity of the barium salt was prepared by treating 25 grams of the substance with barium hydroxide and purifying the barium salt in the same way as before, except that after precipitating the dilute hydrochloric acid solution with alcohol the mixture was allowed to stand for several days

After drying at 125° the following results were obtained on analysis

C, 12.05, H, 2.46, P, 13.83, Ba, 32.19 per cent

C, 11.85, H, 2.32, P, 13.82, Ba, 32.08 per cent

Although the barium is found somewhat low this salt corresponds to the penta-barium salt of the acid,  $C_{20}H_{55}O_{49}P_9$

For  $C_{20}H_{45}O_{49}P_9Ba_5 = 2035$

Calculated C, 11.79, H 2.21, P, 13.71, Ba, 33.76 per cent

The free acid prepared from this salt by the same method as before gave the following results on analysis after previously drying at  $130^\circ$

I C, 16.91, H, 3.96, P, 20.88 per cent

II C, 16.91, H, 3.84 per cent

It appears then that the substance,  $C_{25}H_{65}O_{54}P_9$ , is very sensitive to acids and that when it is kept in contact with even dilute acids for any length of time the elements of one pentose,  $C_5H_{10}O_5$ , are split off

*Brucine salt of the acid,  $C_{20}H_{55}O_{49}P_9$*

While it was impossible to obtain any crystalline salts of the above acid with inorganic bases it gave a crystalline brucine salt of the formula  $C_{20}H_{55}O_{49}P_9(C_{23}H_{26}O_4N_2)_{10} + 30 H_2O$

About 1 gram of the acid was dissolved in a small quantity of water and brucine was then added until the solution showed a slight alkaline reaction. After diluting the solution with 150 cc of alcohol and 30 cc of chloroform, ether was added until a slight permanent turbidity remained. On standing for several days at room temperature in a well closed Erlenmeyer flask the substance separated slowly in long white silky needle-shaped crystals.

In the absence of chloroform or in more concentrated solutions only amorphous white precipitates are obtained.

The crystals were filtered off and washed in a mixture containing equal parts of alcohol and ether and finally in ether and dried in the air. Yield, about 0.5 gram.

The substance is very soluble in water, readily soluble in alcohol but insoluble in ether or chloroform.

Heated in a capillary tube the substance melts at  $196^\circ$ – $198^\circ$  but the melting point is not sharp. On moist litmus paper it shows a strong acid reaction. It loses weight on drying corresponding to  $30 H_2O$ . The dried substance was analyzed.



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0 1364 gram substance lost 0 0124 gram  $\text{H}_2\text{O}$  and 0 1308 gram substance lost 0 0118 gram  $\text{H}_2\text{O}$

0 1240 gram substance gave 0 0694 gram  $\text{H}_2\text{O}$  and 0 2557 gram  $\text{CO}_2$

0 1383 gram substance gave 0 0233 gram  $\text{Mg P}_2\text{O}_7$

0 1190 gram substance gave 6 1 cc of nitrogen at  $16^\circ$  and 746 mm

For  $\text{C}_{20}\text{H}_{45}\text{O}_{49}\text{P}_9$ ,  $(\text{C}_{23}\text{H}_{26}\text{O}_4\text{N}_2)_{10} = 5298$

Calculated C, 56 62, H, 5 94, P, 5 26, N, 5 28 per cent

Found C 56 24, H, 6 26, P, 4 69, N, 5 86 per cent

Calculated for 30  $\text{H}_2\text{O}$  9 24 per cent, found 9 09 and 9 02 per cent

### *Action of dilute sulphuric acid on the barium salt, $\text{C}_{20}\text{H}_{45}\text{O}_{49}\text{P}_9\text{Ba}_5$*

Five grams of the air-dried salt were boiled for one hour under a reflux condenser with 100 cc of  $\frac{1}{2}$   $\text{H}_2\text{SO}_4$ . The reaction mixture was precipitated with slight excess of barium hydroxide, filtered and washed with water. The filtrate was examined as mentioned below.

The barium precipitate was shaken up with 300 cc of 0 5 per cent hydrochloric acid and the insoluble portion filtered off. To the filtrate was added an equal volume of alcohol and the white flocculent precipitate filtered off and washed in dilute alcohol. It was again dissolved in 0 5 per cent hydrochloric acid, precipitated with alcohol, filtered, washed free of hydrochloric acid with dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. Yield, 2 grams. The product was a white amorphous powder. After drying at  $120^\circ$  the following results were obtained on analysis:

Found C, 11 64, H, 2 25, P, 13 95, Ba, 33 26 per cent

This corresponds exactly with the composition of the substance before treatment with  $\frac{1}{2}$   $\text{H}_2\text{SO}_4$ . It is apparent therefore that no partial decomposition takes place.

### *Examination of the filtrate from above*

The excess of barium hydroxide was removed with carbon dioxide and, after filtering, the filtrate was concentrated in vacuum at a temperature of  $35^\circ$ – $40^\circ$  to small bulk, again filtered and finally dried in vacuum over sulphuric acid. There remained 0 08 gram of a slightly amber colored amorphous mass, of weak acid reaction on litmus paper and a slightly acid taste. The aqueous solution

reduced Fehling's solution strongly on boiling and it also gave the orcinic and phloroglucine reactions. The small quantity of the substance prevented any further examination.

In another case 2.5 grams of the same barium salt were boiled with 100 cc of  $\frac{N}{2}$   $\text{H}_2\text{SO}_4$  under a reflux condenser for ten hours. After treating in the same way as above 0.3 gram of unchanged substance was obtained and the filtrate showed exactly the same properties as mentioned above. Attempts to isolate inositol failed.

*Preparation of inositol from the barium salt,  $\text{C}_{20}\text{H}_{45}\text{O}_{19}\text{P}_9\text{Ba}_5$*

Of the same barium salt 2.73 grams and 20 cc of 5N  $\text{H}_2\text{SO}_4$  were heated in a sealed tube for three hours to  $160^\circ$ . There was no pressure noticeable on opening the tube. Some free carbon had separated and the solution was of light brown color. The neutralized solution did not reduce Fehling's solution. The inositol was isolated in the usual way and after recrystallizing from dilute alcohol and ether was obtained in needle-shaped crystals, free from water of crystallization. It gave the reaction of Scherer and melted at  $220.5^\circ$  (uncorrected) which leaves no doubt but that the substance was pure inositol. Yield, 0.73 gram, which is equal to 90 per cent of the total carbon present in the barium salt used. The air-dried substance was analyzed.

0.1649 gram substance gave 0.1038 gram  $\text{H}_2\text{O}$  and 0.2406 gram  $\text{CO}_2$ .

0.1323 gram substance gave 0.0815 gram  $\text{H}_2\text{O}$  and 0.1931 gram  $\text{CO}_2$ .

For  $\text{C}_6\text{H}_8(\text{OH})_6 = 180$

Calculated C, 40.00, H, 6.66 per cent

Found C, 39.80, H, 7.04 per cent

C, 39.80, H, 6.89 per cent

The 0.2 per cent hydrochloric acid extract of bran contains some dissolved proteins. On precipitating with alcohol these are thrown down together with the phosphorus compounds. Their presence makes the subsequent purification difficult, especially the filtrations, because the proteins have been rendered more or less insoluble and form a fine slimy mass which clogs the filter paper to such an extent as to make filtration even by suction extremely tedious.

In order to obviate this, the suggestion was made by Dr. Jordan to first precipitate the bran extract with tannic acid.

The addition of tannic acid was found to cause a voluminous and very fine precipitate which after standing a short time becomes coarser and may then be easily removed by simple filtration. The resulting filtrate is nearly colorless or of light amber color. Alcohol produces in this solution a nearly colorless precipitate which is much more easily purified than the product obtained without first precipitating with tannic acid.

With only this modification some of the substance was prepared from wheat bran. It was found, however, to differ slightly in composition from that obtained by the first method. On analysis the following results were obtained:

C, 19.51, H, 3.09, P, 15.23, Ca, 0.38, Mg, 7.35, K, 2.75, N, 0.57 per cent

On treating this substance with barium hydroxide and purifying the resulting precipitate in the same way as before, the same barium salt was obtained.

For  $C_8H_{11}O_6P_2Ba_2 = 2184$

Calculated    C, 13.73, H, 2.51, P, 12.76, Ba, 31.44 per cent

Found        C, 13.00, H, 2.46, P, 12.47, Ba, 33.00 per cent

The difference in composition of the crude substance must therefore be due to the smaller amount of the nitrogen-containing body which this preparation was found to hold. In the analysis of the crude substance only 0.57 per cent nitrogen was found, whereas the first preparation had four times, and the second preparation two times as much.

*Isolation of the substance as a barium salt directly from the bran extract*

The bran was digested with 0.2 per cent hydrochloric acid overnight. The strained extract was precipitated with tannic acid, filtered and a solution of barium chloride added which caused a small precipitate to separate. An equal volume of alcohol was then added. After settling, the precipitate was filtered and purified as follows. The substance was dissolved in 0.5 per cent hydrochloric acid, precipitated with barium hydroxide in excess, filtered, again dissolved in the same strength hydrochloric acid and then precipitated with alcohol. It was then precipitated a second

time with barium hydroxide and after that precipitated from 0.5 per cent hydrochloric acid with alcohol until the product did not give any reaction with ammonium molybdate

A white amorphous powder was finally obtained. On moist litmus paper it showed a strong acid reaction. After drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide it was analyzed

0.2870 gram substance gave 0.0630 gram  $H_2O$  and 0.1584 gram  $CO_2$

0.3066 gram substance gave 0.0653 gram  $H_2O$  and 0.1700 gram  $CO_2$

0.2632 gram substance gave 0.1437 gram  $BaSO_4$  and 0.1020 gram  $Mg_2P_2O_7$

Found I C, 15.05, H, 2.45, P, 10.80, Ba, 32.12 per cent

II C, 15.12, H, 2.38 per cent

Distilled with 12 per cent  $HCl$ , 0.4736 gram substance gave 0.0071 gram phloroglucid

As will be noticed this compound contains a considerably larger percentage of carbon than any of the previous preparations and a correspondingly low percentage of phosphorus. Calculated on the same basis as before, it would correspond to a molecule with C, 30 or 32. By acting upon this compound with dilute sulphuric acid some reducing body is split off and the salt,  $C_{25}H_{55}O_{64}P_9Ba_5$ , results, identical with that obtained in the first case from the crude substance.

One gram of the above barium salt was digested for about ten minutes with 20 cc. of normal sulphuric acid and heated nearly to boiling. It was then precipitated with excess of barium hydroxide and filtered.

The filtrate was freed from excess of barium hydroxide with carbon dioxide, filtered, and evaporated to small bulk and again filtered. It was then found to reduce Fehling's solution strongly on boiling and to give the orcin and phloroglucine reactions, showing conclusively that a reducing body of some kind had been split off by the action of the sulphuric acid.

The barium precipitate from the above was shaken up with a small quantity of 0.5 per cent hydrochloric acid, filtered and the filtrate precipitated by adding an equal volume of alcohol. After again precipitating in the same manner the substance was filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The substance weighed 0.45 gram. For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

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0.2326 gram substance gave 0.0525 gram  $\text{H}_2\text{O}$  and 0.1208 gram  $\text{CO}_2$

0.1859 gram substance gave 0.1009 gram  $\text{BaSO}_4$  and 0.0804 gram  $\text{Mg}_2\text{P}_2\text{O}_7$

Found C, 14.16, H, 2.52, P, 12.05, Ba, 31.94 per cent

Calculated for  $\text{C}_{25}\text{H}_{35}\text{O}_{54}\text{P}_9\text{Ba}_5 = 2184$

C, 13.73, H, 2.51, P, 12.76, Ba, 31.44 per cent

This substance is therefore identical with the barium salt prepared from the previously isolated crude compound

We are planning to carry out a complete investigation concerning this organic phosphoric acid of wheat bran and its cleavage products. It is desired especially to isolate and identify the reducing bodies formed on cleavage with dilute acid. We also wish to take up the study of the nitrogen-containing substance and beg to reserve the further investigation of these bodies

## STUDIES IN BACTERIAL METABOLISM V

BY ARTHUR I KENDALL AND CHESTER J FARMER

(From the Laboratories of Biological Chemistry and Preventive Medicine and Hygiene, Harvard Medical School)

(Received for publication, July 30, 1912)

Of the organisms investigated in this series, the Flexner and Shiga types of the dysentery bacillus, *Bacillus typhosus* "A" and Paratyphoid  $\alpha$  need no comment, the analytical results are strikingly similar to those previously described with the same species of bacteria, but with different strains<sup>1</sup> The object of studying different strains of the same bacillus from different sources is to confirm our idea that the growth curves should be very similar Such appears to be the case

*B. alcaligenes* is an organism belonging to the typhoid-dysentery group, it differs culturally from these bacteria chiefly because it ferments no sugars The ammonia formation of *B. alcaligenes* is qualitatively and quantitatively like the other members of the group in sugar-free broth The reaction in sugar-free broth is also practically the same In sugar-containing broth, the reaction and ammonia formation is parallel to that in broth containing no carbohydrate This is to be expected, and in a measure this finding is evidence of the correctness of the hypothesis that "fermentation takes precedence over putrefaction" The bacillus cannot attack sugar, hence the sugar acts as a foreign body, taking no part in the metabolic processes of this organism

Cholera "Hamburg" is a typical cholera vibrio which has been on artificial media for several years It is not as active proteolytically as the "Cholera Boston" culture, but it is nevertheless a strong ammonia producer The same sparing action of sugar for protein is exhibited by the Hamburg culture as was the case with the Boston culture

Typhoid "M" was obtained from an intermittent typhoid carrier

<sup>1</sup> This *Journal*, xii, pp 13, 19, 215, 219, 1912

of five years' standing Prior to the isolation of this organism no suspicion of harboring typhoid bacilli was attached to the patient, and no history of secondary cases attributable to him can be obtained Repeated examinations failed to reveal typhoid bacilli before or after the isolation of the strain studied here The organism is typical culturally and morphologically and agglutinates promptly at a dilution of 1 to 3200 with a specific serum The slight irregularities in the growth curve are partly accounted for by the fact that the organism was freshly isolated, having been on artificial media but ten days before it was studied The culture was of undoubted purity The general type of growth curve is like that of the other strains of *B typhosus* studied in this series of experiments

Paratyphoid " $\gamma$  18" deserves special mention it will be seen that this bacillus broke down a considerable amount of protein even in the presence of dextrose which it could use, more so than any organism previously described by us We believe that this bacillus uses up dextrose very rapidly, and that the continually decreasing residual amount of dextrose in the medium is insufficient to fully meet the metabolic requirements of the rapidly growing bacilli This forces them to utilize protein not only for structural purposes, but partly at least for fuel as well Further experiments are in progress to elucidate this phenomenon

*B cloacae* also breaks down protein rather rapidly in the presence of dextrose The reaction curve of this organism is instructive, at first the reaction is distinctly acid, but in a short time the reaction becomes alkaline, and the alkalinity progressively increases We believe that this organism, in common with Paratyphoid " $\gamma$  18," utilizes dextrose with great rapidity, and that the amount of dextrose present in our media (1 per cent) is not large enough to furnish energy except for the first days of growth

H-61 has been described before<sup>2</sup> The diminution of ammonia content, which we have designated as the "negative ammonia phase" is strikingly shown in the table, more than 30 per cent of the ammonia originally present in the medium has disappeared at the end of eight days This negative ammonia phase is seen in the early days of growth in several organisms described previously, although in much less active form

<sup>2</sup> This Journal, xii, p 19, 1912

TABLE I

## Broth E

	DAYS	PLAIN BROTH			DEXTROSE BROTH		
		Free NH <sub>3</sub> as mgms N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc N <sub>2</sub> acid per 100 cc	Free NH <sub>3</sub> as mgms N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction cc N <sub>2</sub> acid per 100 cc
Control		24 50	11 67	-0 50	24 50	11 67	-0 50
B Cloacæ	1	28 00	13 35	-0 25	26 95	12 80	+1 00
	3	30 45	14 50	-0 75	26 60	12 65	-0 25
	5	36 40	17 30	-1 25	30 45	14 50	-1 05
	8	38 50	18 50	-1 50	36 05	17 20	-1 25
Cholera Hamburg	1	27 65	13 15	-0 50	25 90	12 35	+0 50
	3	37 45	17 80	0 00	26 25	12 50	+1 25
	5	34 65	16 50	-1 50	29 75	14 20	+2 25
	8	50 75	24 20	-2 75	28 00	13 35	+2 25
Paratyphoid "γ 18"	1	36 75	17 50	-0 25	36 75	17 50	+0 75
	3	36 75	17 50	0 00	37 75	18 00	+2 25
	5	36 05	17 20	-1 00	36 05	17 20	+2 50
	8	37 80	18 00	-2 00	36 05	17 20	+2 25
Typhoid "M" (Typhoid carrier)	1	26 95	12 80	-0 50	26 25	12 50	+1 75
	3	26 95	12 80	-0 50	28 00	13 35	+1 75
	5	26 60	12 70	-2 00	27 65	13 15	+2 25
	8	28 00	13 35	-1 50	27 05	12 90	+2 75
B dysenteriae, "Flexner"	1	25 90	12 30	-0 25	25 55	12 15	+1 75
	3	25 90	12 30	0 00	27 05	12 30	+2 25
	5	27 30	13 00	-0 25	27 30	12 90	+2 50
	8	27 65	13 15	-1 00	26 95	12 80	+2 50
B alcaligenes	1	28 35	13 50	-0 50	27 65	13 15	-0 50
	3	28 70	13 65	-0 50	29 05	13 80	-1 50
	5	32 55	15 50	-2 00	31 50	15 00	-1 75
	8	33 25	15 80	-1 50	32 90	15 70	-1 25
Typhoid "A"	1	27 30	13 00	-0 50	26 95	12 80	-0 50
	3	28 00	13 30	-0 50	26 95	12 80	+2 50
	5	28 70	13 65	-0 75	26 95	12 80	+2 50
	8	29 05	13 80	-1 25	27 30	13 00	+3 25



TABLE 1—Continued

	DAYS	PLAIN BROTH			DEXTROSE BROTH		
		Free $\text{NH}_3$ as mgms $\text{N}_2$ per 100 cc	Ammonia N Total N	Reaction cc $\text{N}$ acid per 100 cc	Free $\text{NH}_3$ as mgms $\text{N}_2$ per 100 cc	Ammonia N Total N	Reaction cc $\text{N}$ acid per 100 cc
H-61	1	22 75	10 80	-1 00	22 40	10 65	-0 75
	3	19 95	9 50	-2 00	17 50	8 35	-2 00
	5	17 50	8 35	-2 50	16 80	8 00	-2 50
	8	14 70	7 00	-2 75	14 35	6 84	-2 50
B dysenteriae, "Shiga"	1	22 60	10 75	+0 25	25 90	12 35	+0 50
	3	28 00	13 35	-0 50	26 00	12 40	+2 00
	5	29 40	14 00	-0 75	27 30	13 00	+2 00
	8	29 40	14 00	-0 50	26 95	12 80	+2 75
Paratyphoid a	1	26 60	12 70	-0 50	25 55	12 20	+2 00
	3	28 88	13 30	-0 75	26 95	12 80	+2 00
	5	30 10	14 30	-0 75	28 00	13 30	+2 20
	8	31 50	15 00	-1 00	28 00	13 30	+2 75

## STUDIES IN BACTERIAL METABOLISM VI

By ARTHUR I KENDALL AND CHESTER J FARMER

(From the Laboratories of Biological Chemistry and Preventive Medicine and Hygiene, Harvard Medical School )

(Received for publication, July 30, 1912 )

*B. typhosus* and *B. dysenteriae* (Shiga type) are strains of these organisms not previously studied by us. Their growth curves are practically identical with those of other strains described previously.<sup>1</sup> The table shown indicates not only the ammonia and reaction curves in sugar and sugar-free broth respectively, but the daily rate of metabolism of dextrose. The Shiga bacillus shows very distinctly the formation of acid in sugar-free broth, referred to in our last communications.<sup>2</sup> The explanation for this acid formation has been formulated in the fourth article of our series<sup>3</sup> and needs no further comment here.

*B. cloacae*, I and II, show the same initial acidity, followed by a quick return to alkalinity as did the strain described in the preceding communication of this series. On the third day the reaction was strongly alkaline. The rate of decomposition of sugar by these bacteria, amounting to about 75 per cent (of which 63 per cent was used up the first twenty-four hours), explains the reason for this terminal alkaline reaction, the findings are in accord with the opinion expressed in the previous communication upon this phenomenon.

Paratyphoid  $\gamma$  16 and 4 are strains similar culturally to Paratyphoid  $\gamma$  18 described previously. They produce considerable amounts of ammonia, even in the presence of dextrose, thus agreeing essentially with the strain mentioned above in this respect. The reaction, however, becomes progressively acid. 1.

<sup>1</sup> This *Journal*, xii, pp 13, 19, 215, 219, 1912

<sup>2</sup> *Ibid*, xii, p 215, 1912

<sup>3</sup> *Ibid*, xii, p 219 1912

TABLE I

## Broth F

	DAYS	PLAIN BROTH			DEXTROSE BROTH			DEXTROSE USED UP
		Free NH <sub>3</sub> as mgms N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction to $\frac{1}{2}$ acid per 100 cc	Free NH <sub>3</sub> as mgms N <sub>2</sub> per 100 cc	Ammonia N Total N	Reaction to $\frac{1}{2}$ acid per 100 cc	
Control		34 30	per cent 16 35	+0 25	34 30	per cent 16 35	+0 25	per cent
B Typhosus	1	31 15	14 85	-0 25	31 50	15 00	+2 75	6 85
	3	32 20	15 35	-0 25	31 85	15 15	+3 25	28 80
	6	44 10	19 50	-1 50	32 90	15 65	+3 25	41 00
	8	44 30	23 00	-1 50	34 30	16 35	+3 75	47 30
B Cloacae I	1	32 20	15 35	-0 50	30 10	14 30	+0 75	63 00
	3	42 70	20 30	-1 50	37 10	17 65	-0 75	74 80
	6	49 70	23 70	-3 00	50 40	24 20	-1 50	77 40
	8	49 70	23 70	-3 25	62 30	29 65	-2 25	82 20
Paratyphoid " $\gamma$ 4"	1	42 00	20 00	-1 00	37 10	17 65	+1 75	41 10
	3	57 40	27 35	-2 00	40 95	19 50	+2 75	64 40
	6	84 70	40 30	-3 25	42 00	20 00	+2 50	63 00
	8	95 20	45 40	-4 25	44 45	21 20	+3 50	72 00
Shiga Bacillus	1	31 85	15 15	+0 25	30 80	14 70	+2 00	23 30
	3	35 00	16 65	+0 75	32 90	15 65	+3 25	28 80
	7	37 80	18 00	$\pm$ 0 00	32 20	15 35	+2 75	47 20
	9	40 95	19 50	-0 25	34 65	16 50	+2 75	50 70
Paratyphoid " $\gamma$ 16"	1	45 50	21 70	$\pm$ 0 00	48 65	23 15	+0 50	38 40
	3	57 40	27 30	-1 25	53 20	25 40	+2 75	63 00
	7	61 95	29 50	-2 50	55 65	26 50	+3 25	68 40
	9	69 65	33 20	-2 75	59 85	28 50	+4 25	77 40
B Cloacae II	1	31 85	15 15	$\pm$ 0 00	30 10	14 35	+1 00	66 40
	3	42 00	20 00	-0 75	35 00	16 70	-2 00	74 80
	7	46 20	22 00	-3 25	53 20	25 40	-2 50	74 80
	9	53 90	25 70	-3 75	57 40	27 30	-2 75	76 80

teria grow with unusual rapidity, and it is possible that the rapidity of growth necessitates a considerable amount of protein breakdown to supply the necessary nitrogen for structural purposes. These strains also utilize about 72 per cent of the dextrose of the medium. This utilization of dextrose suggests a large fuel requirement, the rather uniform breakdown of the sugar contrasting in this respect with the large initial breakdown of dextrose in the case of the various strains of *B. cloacae*, where, it will be remembered, the breakdown of sugar was largely within the first twenty-four hours of growth. In the case of Paratyphoid  $\gamma$ , we are inclined to believe that the utilization of dextrose is far less per unit time per organism than is the case with the various strains of *B. cloacae*, where the sugar largely disappears within the first day of growth, forcing the bacteria to derive at least part of their fuel from nitrogenous products.



# THE RÔLE OF GLIADIN IN NUTRITION<sup>1</sup>

BY THOMAS B OSBORNE AND LAFAYETTE B MENDEL,

WITH THE COÖPERATION OF EDNA L FERRY

*(From the Laboratories of the Connecticut Agricultural Experiment Station  
and the Sheffield Laboratory of Yale University, New Haven, Connecticut )*

(Received for publication, July 31, 1912 )

Our notions regarding the relation of the food proteins to tissue proteins, and the rôle of proteins in nutrition have experienced radical changes in recent years. Side by side with the increasing evidence of distinct structural differences between the albuminous compounds of different origin and the chemical dissimilarity which may even characterize two proteins derived from a common source, such as some particular seed, has arisen the well founded conviction that it is impossible to develop marked changes in the character of the tissues of animals correlated with the character of the food ingested. Whatever may be the source, or chemical make-up, of the latter previous to its involvement in the nutritive processes, the resulting tissue cells and fluids remain characteristic and specific for the species. "Der Artcharakter wird durch die Art der Ernährung nicht beeinflusst" (Abderhalden).

How this possibility of the fixity of the tissues in the midst of diversity of food types results is made apparent by the newer knowledge respecting the rôle of digestion in nutrition. The structural peculiarities which determine the individuality of the proteins are lost by the digestive process, hence we have ultimately to deal with the fragments of the original complexes in the problems pertaining to nutrition. Our food stuffs are currently assumed to leave the alimentary tract largely, if not entirely, in the form of

<sup>1</sup>The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

the so-called amino-acid "Bausteine" It is these which become our immediate concern in the intermediary problems of metabolism that result in the construction or renewal of the specific body protein Quoting Abderhalden "Unsere Körperzellen erfahren niemals, welcher Art die aufgenommene Nahrung war"

In the organism proper the proteins, as such, may be responsible for various physiological functions "At present we cannot fully comprehend the rôle of the proteins, but we must assume that many of the enigmatical properties of living matter depend on this activity of *intact* protein molecules We can obtain some idea of the possible variety in the combinations of the protein Bausteine by recalling the fact that they are as numerous as the letters in the alphabet which are capable of expressing an infinite number of thoughts Every peculiarity of species and every occurrence affecting the individual may be indicated by special combinations of protein Bausteine, that is to say by specific proteins Consequently we may readily understand how peculiarity of species may find expression in the chemical nature of the proteins constituting living matter, and how they may be transmitted through the material contained in the generative cells"<sup>2</sup> As one of us has written earlier "The results of my work have shown that no two seeds are alike in their protein constituents, and that those proteins which appear to be alike are found only in seeds that are botanically closely related As I have elsewhere pointed out, it would seem that these differences in the reserve food substances of the endosperm must have an important bearing on the character of the developing embryo which derives its first food from them This food substance, and the embryo as well, are the final products of the series of chemical changes which led to their formation When the embryo begins its development it finds at hand a definite food, which for each individual of the same species is the same, but for the individuals of different species is different Each member of a species begins its independent life under similar chemical conditions, but under chemical conditions which are different from those of every other species When, therefore, each individual plant reaches that stage of development at which its organs of assimilation are able to furnish it with nutri-

<sup>2</sup> Kossel Lectures on the Herter Foundation The Proteins *Johns Hopkins Hospital Bulletin*, xxiii, p 76, 1912

ment from its external surroundings, it is highly probable that its chemical processes have already been established along definite lines which it must follow throughout the rest of its life ”<sup>3</sup>

In the preliminary processes of metabolism, however, the character of the amino-acid fragments apparently assumes a dominating importance. The modern chemistry of the proteins has disclosed the fact that the variations between the different albuminous compounds in respect to their Bausteine may be both quantitative and qualitative in character. This has raised the question of the relative physiological value of unlike proteins. “The fact that so many of the vegetable proteins, which serve extensively as food, have been shown, by our present investigation, to yield such different proportions of the various nitrogenous decomposition products, as compared with the animal proteins, makes it a matter of the greatest interest and importance to know something more of the processes involved in this synthesis ”<sup>4</sup>

Whether protein can be suitably utilized when administered in its completely digested or abiuiret form as well as in its natural condition need not concern us here, since the possibility of maintaining individuals in satisfactory nutritive balance, at least for a not inconsiderable time, on an intake made up exclusively of Bausteine has been demonstrated. It would seem, therefore, as if the problem of replacing the larger protein complexes by their elementary constituent fragments had been to a certain extent solved. If we assume, in harmony with some of the prevailing views of metabolism, and notably that supported by Abderhalden, that the animal must construct its tissue proteins from the amino-acid fragments which are furnished by protein hydrolysis, it is obvious that deficiencies in quantity in the Bausteine or a lack of one or more of them must lead to serious nutritive disturbances. The chemical fixity of the tissues under widely differing nutrient environment points in the same direction. Abderhalden has maintained that, so long as there is no evidence that amino-acids can readily experience a transformation into one another in the organism, the extent of protein construction in the body must be

<sup>3</sup> Osborne *Proc Soc Exp Biol and Med*, v, p 105, 1908

<sup>4</sup> Osborne and Harris *Jour Amer Chem Soc*, xxv, p 323, 1903

<sup>5</sup> Cf Abderhalden *Synthese der Zellbausteine in Pflanze und Tier*, Berlin, 1912



limited by the amino-acid which is present in the smallest relative amount in our intake. The fact that certain proteins, such as gelatin and zein, which are notably defective in respect to the number of the amino-acids which they yield, are unable by themselves to promote nutritive equilibrium and supply the nitrogenous needs of the diet might be quoted in support of the views mentioned above. If Abderhalden's hypothesis regarding the nature of protein metabolism is correct it follows that those food proteins which approach most nearly to the tissue proteins in their amino-acid make-up should most easily supply the protein needs of the animal. Michaud<sup>6</sup> has undertaken to demonstrate, in accord with this, that the protein minimum of dogs can be maintained at a lower level when the intake is in the form of dog tissue than in the form of proteins differing widely therefrom in their chemical make-up, yet the investigations heretofore recorded with these proteins lead to the belief that they are, at least to some degree, utilized as food by the animal, even when they are fed as the sole source of nitrogen. Some of these proteins lacking one or more of the cleavage products known to be necessary for the formation of the proteins of the animal body are of relatively high efficiency in preventing loss of body nitrogen due to endogenous metabolism, although they are insufficient for growth.<sup>7</sup> It is evident that "the processes of replacing nitrogen degraded in cellular metabolism are not of the same character as the processes of growth. It seems also to be a necessary conclusion that the processes of cellular catabolism and repair do not represent a series of chemical changes involving the destruction and reconstruction of an entire protein molecule."<sup>8</sup> Regarding the necessity of distinguishing carefully between maintenance, repair and growth in nutrition we shall have more to say later. Undoubtedly the failure to bear these distinctions in mind has led to much confusion in the past. Furthermore, investigators have heretofore been so largely concerned with the functions of proteins as a whole in important biological processes that the possibility of their individual participation

<sup>6</sup> Michaud *Zeitschr f physiol Chem*, lxx, p 405, 1909, cf also Frank and Schittenhelm *ibid*, lxx, p 99, 1910, lxxiii, p 157, 1911

<sup>7</sup> Cf Osborne and Mendel Carnegie Institution of Washington, Publication 156, pt 11, 1911, also *Zeitschr f physiol Chem*, 1912 (in press)

<sup>8</sup> McCollum *Amer Journ of Physiol*, cxix, p 215, 1911

and use has been generally overlooked. As Kossel has lately said "Hitherto the appearance of protein Bausteine in the living organism has always been ascribed to protein decomposition. But this supposition is unjustified. We must rather assume that these Bausteine may appear and disappear in the body without at any time forming part of a protein molecule. And further we may suppose that only under certain circumstances, for definite physiological purposes, are these independent groups stored in a collected form—the protein substances"<sup>9</sup>

Attempts have been made at various times in the past to perfect the so-called abnormal or incomplete proteins by adding to them in the diet one or more amino-acids which are known to be lacking from the complex. This is true of studies made with gelatin—which yields no tyrosine, tryptophane or cystine—and with zein,—a protein which yields no tryptophane, and from which no lysine or glycocoll can be obtained. These trials have, all in all, not been very satisfactory. Other experiments in which an amino-acid, such as tryptophane, has been intentionally eliminated from the food mixture have speedily exhibited a nutritive defect in the dietary. In any event it seems clear, from such evidence as is available at the present time, that the cyclic compounds, tyrosine, phenylalanine, histidine and tryptophane, are indispensable for the welfare of the organism. Indeed W A Osborne has expressed the view that the essential difference between the animal and the plant organism lies in their respective ability or inability to synthesize substances of the cyclic type. Cyclopoiesis, according to him, is a property exhibited solely by the vegetable organism.

Are the other amino-acids equally indispensable? At the present moment it is impossible to give any definite answer to the question as to whether an amino-acid like leucine, for example, can be replaced by alanine, or any other closely related form. In one case, in any event, the possibility of a synthesis of an amino-acid *de novo* in the animal organism has been admitted. Prolonged feeding experiments with casein from which glycocoll has not been obtained, as well as the enormous production of glycocoll for the hippuric acid synthesis after the administration of benzoic

<sup>9</sup> Kossel Lectures on the Herter Foundation. The Proteins. *Johns Hopkins Hospital Bulletin*, VIII, p 76, 1912

acid<sup>10</sup>—a production out of all proportion to the assumed content of preformed glycocoll in the food intake, or the body tissues themselves—leave little doubt of the capacity of the animal cell to synthesize at least one amino-acid

For evidence of the formation of amino-acids more complex than glycocoll, the recorded experiments with gliadin must be taken into consideration. This substance, an alcohol-soluble protein of the prolamine type, possesses a special interest in that it yields so little of the diamino-acid, lysine, as well as of glycocoll, that these have not heretofore been obtained from it by the usual analytical methods. It furthermore contains a relatively small proportion of both arginine and histidine, and extremely large proportions of glutamic acid and ammonia yielding groups. Its ready digestibility has been demonstrated repeatedly<sup>11</sup> in contrast to the greater resistance of the "abnormal" protein zein. Henriques<sup>12</sup> reported that he kept rats in nitrogenous equilibrium on a diet in which gliadin constituted the sole form of nitrogenous intake, although he failed when the tryptophane-free zein was used. Abderhalden and Funk<sup>13</sup> were similarly successful with gliadin fed to dogs. They state, however, that in one case the preparation of gliadin fed by them contained 0.35 per cent of lysine, and they intimate that the nutritive equilibrium secured by Henriques on a diet containing gliadin as the sole protein was due to an impure preparation. The question of lysine synthesis in the body is expressed by Rona<sup>14</sup> as follows: "Das Problem ist also durch die Versuche von Henriques noch nicht gelöst, hingegen sprechen alle unsere Erfahrungen dafür dass die Aminosäuren, Glykokoll ausgenommen, im Organismus nicht neugebildet werden." In other experiments Henriques and Hansen<sup>15</sup> have stated that they were able to get rats into a state of nitrogenous equilibrium on a diet containing the nitrogen solely in the form of the mono-amino fraction of a digest. Here too, as in the experiments with

<sup>10</sup> Cf. Magnus-Levy *Biochem Zeitschr*, vi, p. 523, 1907; Ringer *this Journal*, x, p. 327, 1911; Epstein and Bookman *ibid*, x, p. 353, 1911.

<sup>11</sup> Cf. Mendel and Fine *this Journal*, x, p. 303, 1911.

<sup>12</sup> Henriques *Zeitschr f physiol Chem*, lx, p. 105, 1909.

<sup>13</sup> Abderhalden and Funk *ibid*, lx, p. 418, 1909.

<sup>14</sup> Rona *Oppenheimer's Handbuch der Biochemie*, iv, pt. 1, p. 550.

<sup>15</sup> Henriques and Hansen *Zeitschr f physiol Chem*, xliii, p. 417, 1904, and xlv, p. 113, 1906.

ghadin, a synthesis of nitrogenous compounds of the type precipitable by phosphotungstic acid must be assumed if the nutritive equilibrium of the experimental animals was at all adequate. It will be seen, therefore, that the problem of di-amino synthesis has heretofore largely hinged upon the validity of the work of Henriques.

The situation has been summed up by Rona in these words: "Vorläufig müssen wir also daran festhalten, daß eine Ueberführung einer Aminosäure in eine andere, bezw. eine Neubildung einer Aminosäure (Glykokoll ausgenommen) im tierischen Organismus nicht stattfindet."<sup>15</sup>

#### EXPERIMENTAL PART

Employing the methods which we have developed in recent years in connection with our feeding experiments with isolated food substances<sup>17</sup> we have accumulated a large number of data which refer directly to the nutrient rôle of ghadin in the animal organism. Inasmuch as we have succeeded, by the application of care in the management of the rats, by furnishing suitable hygienic environment and appropriately selected diet, in maintaining these animals in good nutritive condition on mixtures of isolated food stuffs over periods of more than 500 days, we believe that some of the criticisms which have been aimed at experiments carried out on rats are thereby met. The guiding considerations which have led to the special proportions of nutrients, etc., in the food mixtures reported below have been discussed in some detail in our previous publications.<sup>18</sup> The upshot of our trials has been the demonstration that the ghadins of wheat and rye, as well as the closely related alcohol-soluble hordenin of barley—all of which are similar in the proportion of their Bausteine—suffice for the maintenance of rats without growth.

<sup>15</sup> Rona, *Oppenheimer's Handbuch der Biochemie*, iv, pt. 1, p. 550.

<sup>17</sup> Osborne and Mendel, Carnegie Institution of Washington, Publication 156, pts. 1 and 11, 1911, *Zeitschr. f. biol. Technik u. Methodik*, 11, p. 313, 1912, and *Zeitschr. f. physiol. Chem.*, 1912 (in press).

<sup>18</sup> Osborne and Mendel, Carnegie Institution of Washington, Publication 156, pt. 11, 1911, *Science*, N. S., xxiv, p. 722, 1911, *Zeitschr. f. physiol. Chem.*, 1912 (in press).

*Preparation and composition of gliadin*

The gliadin used for these feeding experiments was made from very thoroughly washed wheat gluten from which all proteoses and other water-soluble proteins had been removed as completely as possible. The alcoholic extract of this gluten was filtered water-clear, thereby separating any suspended glutenin or other proteins insoluble in 70 per cent alcohol. After concentrating the alcoholic extract the residual gliadin was dissolved in alcohol, and its solution poured in a thin stream into a very large volume of cold water, thereby removing any water-soluble substance which might possibly be set free when the gluten was dissolved. The precipitated gliadin was again dissolved in alcohol, and its syrupy solution poured into a very large quantity of absolute alcohol, and thus precipitated as a coherent mass. This was then digested with fresh quantities of absolute alcohol, and finally with ether and was easily reduced to a powder. After drying in the air, the gliadin thus obtained formed a snow white powder which was completely soluble in 70 per cent alcohol. It is difficult to see how gliadin, thus prepared, can contain any other proteins than those soluble in alcohol, or how any purer preparation could be made.

As it was of the greatest importance to know whether or not this gliadin was entirely free from lysine, we made a very careful examination of two portions of 100 grams each, according to the method of Kossel and Kutscher, with which we have had extensive experience. The result was in each case entirely negative, corresponding with our earlier experience, as well as with that of Kossel and Kutscher and of Abderhalden. However, in view of the relatively considerable precipitate produced by phosphotungstic acid, it seemed possible that some lysine might be contained therein, under conditions which rendered its separation as the picrate difficult. This seemed the more probable in view of our previous experience in attempting to isolate lysine as the picrate directly from the products of the hydrolysis of casein. In this attempt we obtained less than one-half as much as by Kossel and Kutscher's method, thus showing the effect of the presence of other amino-acids.

We accordingly made renewed efforts to obtain lysine picrate from our solutions. In one case fractional precipitation with phosphotungstic acid was employed without success. In the other

case the alcoholic solution to which picric acid had been added was divided into two parts, one of which was allowed to evaporate slowly until nearly dry. The semicrystalline residue was extracted with alcohol. The insoluble residue when recrystallized gave 0.2 gram of lysine picrate. The other half of the solution was neutralized with acetic acid and allowed to evaporate slowly until a considerable quantity of free amino-acids separated. These were filtered out and washed with alcohol. The alcoholic filtrate was neutralized with sodium hydroxide and treated with sodium picrate, whereupon a small precipitate of lysine picrate formed, which when recrystallized weighed 0.23 gram. We thus obtained 0.43 gram of lysine picrate from 100 grams of gliadin corresponding to 0.15 per cent of lysine in this preparation.

Whether or not this represents all of the lysine in this gliadin cannot, of course, be determined, but our previous experience with casein has convinced us that it is very difficult to separate all of the lysine picrate from solutions containing other amino-acids. Whether the presence of this lysine is to be ascribed to contamination of our preparation of gliadin with other proteins, or to the presence of a small amount of lysine in gliadin, is likewise difficult to determine. All we can say is that we do not know how any purer preparation of the substance heretofore known as gliadin can be made, and our present opinion is that future investigations will show that gliadin does in fact yield a little lysine.

The question is, therefore, raised: can the absence of any amino-acid from any protein be assumed solely because it cannot be separated by direct crystallization? In our opinion it cannot be so assumed. The known difficulty encountered in trying to thus separate *all* of any of the amino-acids from mixtures of them supports this view, as does also the experience of Osborne and Jones and the more recent experience of Abderhalden. Both of these investigations showed that less than one-half the glycocoll, alanine or aspartic acid could be recovered from mixtures containing known quantities of pure amino-acids.

Whether or not gliadin is actually deficient in glycocoll or lysine, we do know from incontrovertible evidence that it yields relatively very little glycocoll, arginine, histidine or lysine and extremely large quantities of glutamic acid, proline and ammonia. Gliadin, therefore, has a unique constitution, very different from

the tissue proteins of animals, as well as from most of the other proteins which are commonly present in the foods of men and animals. We should consequently expect to find the value of gliadin in nutrition to be different from that of other proteins which yield amino-acids in proportions corresponding more closely with those obtained from proteins of animal origin. Accordingly we have made a large number of prolonged feeding trials on both mature and growing rats, with the results described in the following pages.

*Maintenance experiments with grown rats*

The illustrative protocols which are presented in graphic form are largely self-explanatory. The abscissae of the curves represent days and the ordinates actual body weight (solid line) or food-intake (dotted line) in grams. In the charts for ungrown animals the average (normal) curve of growth, plotted from body weight data available for normally growing animals of the same sex, is represented by a broken line for comparison. The food-intake curve is plotted from the weights of food eaten per week. Where numbers are marked on body weight curves they indicate the time at which changes in the character of the feeding were instituted.

In Charts 1, 2 and 3 are represented the results of prolonged maintenance trials with full grown rats in which gliadin formed the sole nitrogenous intake.<sup>19</sup> These experiments far exceed the longest records of trials in any way comparable with our own which have been reported in the literature. Henriques' records, for example, extend at best over only 23 days.<sup>20</sup> A study of the dietaries quoted in connection with these charts will show that, for long periods, in several of our experiments, there was no possibility of the inclusion of any other protein than the gliadin itself in the make-up of the food, except in the very small quantity of feces supplied during period 2.<sup>21</sup> Thus rat 130, Chart 1, was

<sup>19</sup> Some of the earlier portions of the charts in this paper have already been published elsewhere.

<sup>20</sup> Henriques *Zeitschr f physiol Chem*, lx, p 105, 1909.

<sup>21</sup> See Osborne and Mendel. Carnegie Institution of Washington, Publication 156, pt. II, p. 60 for discussion of the effect of feces thus fed.

fed for 290 days on a food entirely free from any other protein than gliadin, before his condition became such as to render a change in his diet necessary. That the failure to be longer maintained in a satisfactory condition was not due to deficiencies in the gliadin is proved by the rapid recovery of health and weight when the non-protein constituents of the food were changed by replacing the inorganic constituents and a part of the carbohydrate with "protein-free milk"<sup>22</sup>. A similar condition is shown by rat 134, Chart 2, but in this experiment the decline in weight occurred much earlier and the change in the non-protein constituents of the food had to be made after only 72 days. Rat 147, Chart 3, was kept on the original gliadin food for 256 days, but at that time its loss of weight and physical condition was such that it could only be restored by changing the protein to casein. Later (see period 6), the failure to thrive on the original gliadin food was completely remedied by the addition of "protein-free milk" to the diet.

A possible criticism of these experiments concerns the residual content of milk protein in the "protein-free milk". Such analyses as we have made have indicated that the extent of this contamination cannot exceed 0.6 per cent of the entire food mixture—a quantity of "normal protein" far too small, as we have convinced ourselves by other studies directed to this point, to meet the nutrient deficiency of gliadin in respect to growth. However, the experiments which have been conducted without the use of the protein-free milk bear direct testimony in favor of the conclusion that possible traces of contaminating milk protein cannot in any way explain the satisfactory maintenance of our animals, but that some other substance than the protein is the cause of rapid recovery induced by the addition of the protein-free milk.

In the light of such long continued experiments, extending as they do over a very considerable portion of the natural life of an animal whose longevity has been estimated at about three years, one must accept these observations as evidence that, so far as maintenance is concerned, the protein of the food can differ very widely in its amino-acid make-up from the tissue proteins of the animal without affecting the well being of the latter.

<sup>22</sup> See *ibid* p 80



*Maintenance experiments with growing rats—failure to grow*

The following charts illustrate the inability of wheat gliadin and other prolamines to promote growth under dietary conditions in which other single proteins have been eminently satisfactory. In Charts 4, 5 and 6 the curves of growth with casein, edestin, and glutenin will be found to correspond closely, during the first 100 days, to those observed on animals receiving mixed food.

The contrast of the trials with gliadin are striking in the extreme, and the results are the same if the alcohol-soluble horden from barley or the gliadin from rye are used. We have tested the gliadin of wheat,<sup>23</sup> Charts 7, 8 and 9, of rye,<sup>23</sup> Charts 10 and 11, and the similarly constituted horden of barley,<sup>23</sup> Charts 12 and 13. The results are the same, whether the trials be made at a very early age (compare Chart 7) or somewhat later (compare Chart 12). In corroboration of the statement that the results described represent true maintenance without growth, we present two experiments, one with gelatin, Chart 14, and one with zein, Chart 15, which show by contrast the failure of maintenance when an absolutely inadequate protein, like those mentioned earlier, forms the nitrogenous constituent of the food intake.

The youthful appearance of animals thus maintained without growth corresponds in every respect, so far as external characters go, with the size rather than the age of the animal.<sup>24</sup> That the failure to grow is in nowise attributable to any toxicity or inhibitory property of the specific proteins used is shown by experiments (see Charts 16 and 17) in which the addition of a small proportion of an "adequate" protein has sufficed to induce noteworthy growth. To determine whether growth could in any way be induced by largely increasing the content of the "inadequate" protein in the food mixture, special experiments were undertaken (see Charts 18, 19 and 20). The relative variations in body weight in relation to the larger protein intake in the two series are too small to be of marked significance.

<sup>23</sup> The preparation and properties of these proteins are given by Osborne and Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, II, 1909.

<sup>24</sup> Photographs of some of our ungrown animals maintained on gliadin will be found in Osborne and Mendel. Carnegie Institution of Washington, Publication 156 pt. II, 1911.

Aside from their interest in furnishing a physiological differentiation between various proteins, as exemplified in the capacity or failure of maintenance, and the capacity or failure of growth, these experiments have a large field of interest in presenting a method whereby the effective stunting of animals can be induced at any stage in the normal period of growth. We have as yet not determined the possible alterations in the histological make-up of the organs and tissues which may be correlated with the suppression of growth. There is much in the recent literature on infantilism which suggests that the dwarfing may be secondary to defects or alterations in organs, such as the ductless glands. One point alone may be emphasized here, namely, that the capacity to grow is by no means lost even after very prolonged periods of stunting with the gliadin diet. This is shown in Chart 7 which exhibits satisfactory growth on a suitable dietary after a continuous suppression of growth lasting 277 days, when the animal was 314 days old—an age at which normally little or no growth takes place<sup>25</sup>

#### *Gliadin and gestation*

Long continued feeding with gliadin as the sole source of nitrogen by no means impairs the capacity of the animal to produce healthy young and suitably nourish them. The two animals whose records are presented in Charts 21 and 22 were paired and the female (Rat 129) gave birth to a litter of four at the end of 178 days on the gliadin food mixture. The young rats whose growth records are reproduced in Charts 23, 24, 25 and 26 were nourished satisfactorily by the mother during the first month of their existence, in so far as one can judge by their increase in weight, in comparison with that of normally reared rats. At the end of 30 days, three rats were removed from the mother and put upon diets of casein food, edestin food and milk food respectively. The fourth animal was allowed to remain in the cage with the mother whose sole source of nutriment was the original gliadin food mixture. It will be noted from the records that whereas the three removed animals manifested a normal growth on their new dietaries, which had likewise proved adequate

<sup>25</sup> See also charts cxx, cxxi, cxxii and cxxiii, Publication 156, pt. II, Carnegie Institution of Washington

for growth in many other instances, the rat kept with the mother began to evince a failure to grow at about the period (30 days) when young rats are wont to depend upon extraneous food for nourishment. In the present case this means that *the young animal, forced to depend upon the gliadin food mixture in place of the milk of its mother, showed the typical failure to grow on the "inadequate" diet upon which the mother had not only been maintained but had actually produced young and secreted milk sufficient in quantity and quality to induce normal growth in her offspring*. No doubt can remain, we believe, that in this experiment, in which there has unquestionably been a renewal, or new formation, of body tissue, very large in proportion to the original weight of the mother animal, there must have occurred a synthesis not only of the "Bausteine" deficient in the protein intake, but likewise of tissue and milk components like the nucleic acids (with their content of purines, pyrimidines and organically combined phosphorus), and phospho-proteins, like casein, etc., which were completely missing in the special food intake that had formed the sole food of the mother during several months. Unless one were prepared to maintain a profound alteration in the chemical make-up of this "gliadin family" it must be admitted that synthesis in animal nutrition has here been demonstrated in a striking manner.

We have elsewhere<sup>26</sup> taken cognizance of the possible rôle of alimentary bacteria in furnishing some of the components which may be deficient in the dietary. These synthetic organisms may well be able to build new amino-acids out of a variety of substrates, and the possibility is thereby suggested of the production, through bacterial intervention, of complexes missing or deficient in the original food intake. We can hardly regard this possibility as an explanation of the ability of animals to be maintained on the abnormal proteins, gliadin and hordein, otherwise there is no apparent reason why they should not likewise be maintained by zein or gelatin, nor why growth should not also be possible with every digestible protein through the intervention of the bacterial protein complexes manufactured. It is more likely that growth

<sup>26</sup> Cf. Osborne and Mendel. Carnegie Institution of Washington, Publication 156, pt. 11, p. 61, 1911.

hinges on the intervention of some protein complex not essential for the endogenous metabolism of the individual

The unique features of growth and maintenance on the special proteins here considered serve to emphasize the fact that maintenance experiments alone cannot suffice to solve the problem of the full biochemical value of dietaries. Nutrition involves an ensemble of processes which are determined or modified by factors whose real significance is only beginning to reveal itself. No method of study involving well controlled conditions need be cast aside, but hasty judgment formed as the result of brief feeding trials on larger animals containing an abundant reserve supply must henceforth be accepted with extreme caution.

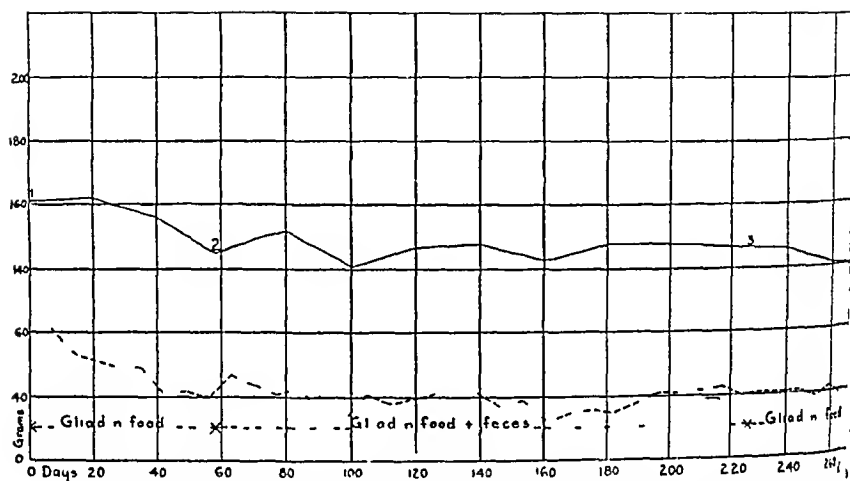
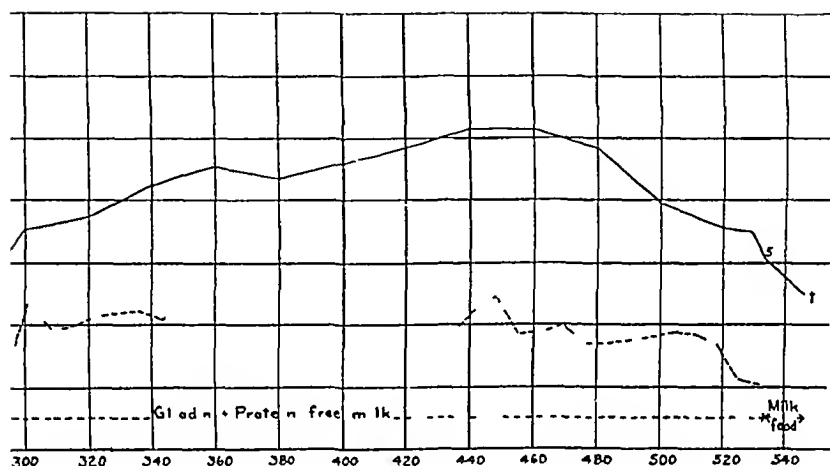


CHART 1, RAT 130 ♀, shows long continued maintenance on a diet containing gliadin, feeding by diseased lungs and a large parasite, over 40 cm long, encysted in the liver.

The diet during the different periods is shown below. During period 2 above the effect of this is discussed in Publication 156, pt II, p 61, Carnegie Institution of Washington.

#### PERIODS 1 2 AND 3

	per cent	
Gliadin (wheat)	18.0	Gliadin (wheat)
Starch	29.5	Protein free milk
Sucrose	17.0	Starch
Agar	5.0	Agar
Salt mixture I	2.5	Lard
Lard	28.0	
	100.0	



sole protein The animal's life was terminated after 546 days of experimental  
of air-dry feces from rats on a mixed diet was given each week The possible

PERIOD 5		
<i>per cent</i>		<i>per cent</i>
18 0	Milk powder	60 0
28 2	Starch	12 0
20 8	Lard	28 0
5 0		100 0
28 0		
<u>100 0</u>		

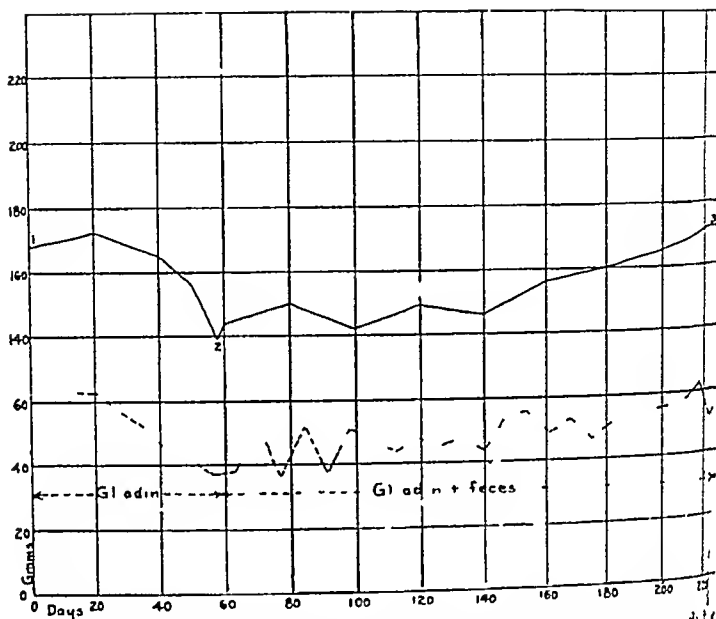
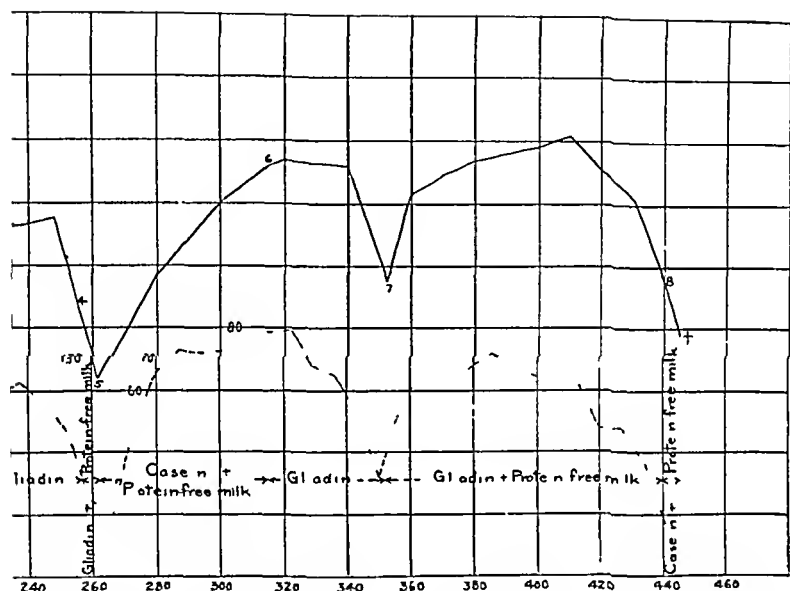


CHART 3, RAT 147 ♀, shows long continued maintenance on a diet with lung disease after 445 days of experimental feeding

The diet during the different periods is shown below During legend on Chart 1

PERIODS 1 2 3 AND 6

	per cent	
Gliadin (wheat)	18 0	Gliadin (wheat)
Starch	29 5	Protein-free milk
Sucrose	15 0	Starch
Agar	5 0	Agar
Salt mixture I	2 5	Lard
Lard	30 0	
	100 0	



obtaining gliadin as the sole protein. The animal's life was terminated by  
 and 2 a small quantity of feces from rats on a mixed diet was supplied. See

PERIODS 4 AND 7

per cent

18.0	Casein (cow's milk)
28.2	Protein free milk
20.8	Starch
5.0	Agar
28.0	Lard
100.0	

PERIODS 5 AND 8

per cent

18.0
28.2
23.8
5.0
25.0
100.0



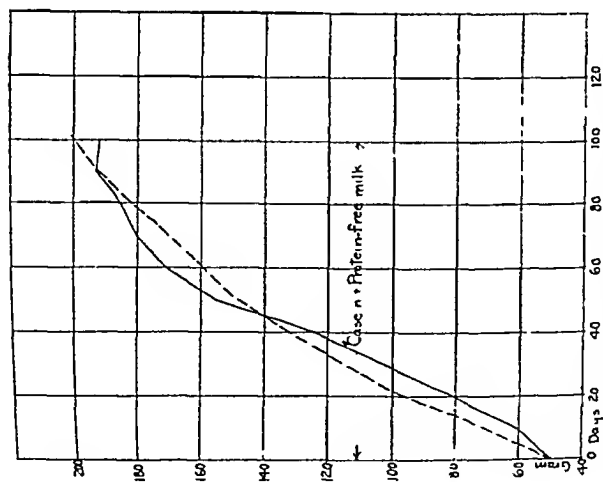


CHART 4, RAT 268 ♂, shows normal growth on a diet containing casein as its sole protein

The diet was

	per cent
Casein (cow's milk)	18.0
Protein free milk	28.2
Starch	23.8
Agar	5.0
Lard	25.0
	100.0

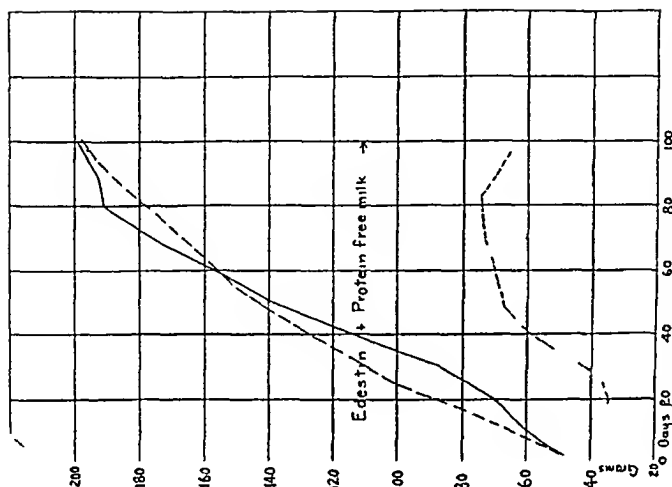


CHART 5, RAT 375 ♂, shows normal growth on a diet containing edestin as its sole protein

The diet was

	per cent
Edestin (hempseed)	18.0
Protein free milk	28.0
Starch	19.0
Agar	5.0
Lard	30.0
	100.0

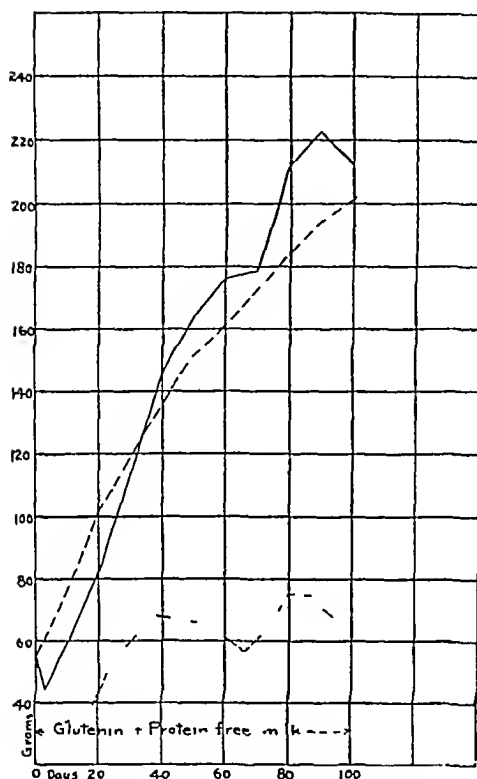


CHART 6, RAT 284 ♂, shows normal growth on a diet containing glutenin which, together with an approximately equal quantity of gliadin forms about 80 per cent of the proteins of the wheat kernel

The diet was

	per cent
Glutenin (wheat)	18 0
Protein free milk	28 2
Starch	23 8
Agar	5 0
Lard	25 0
	<hr/> 100 0

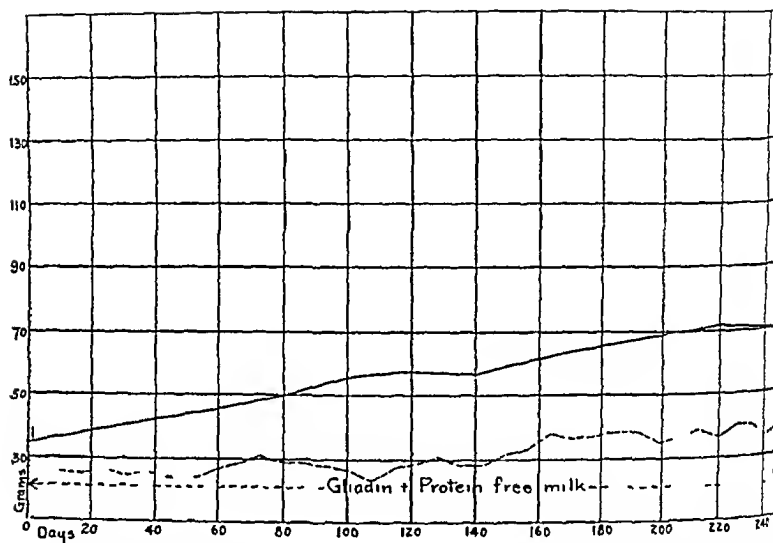
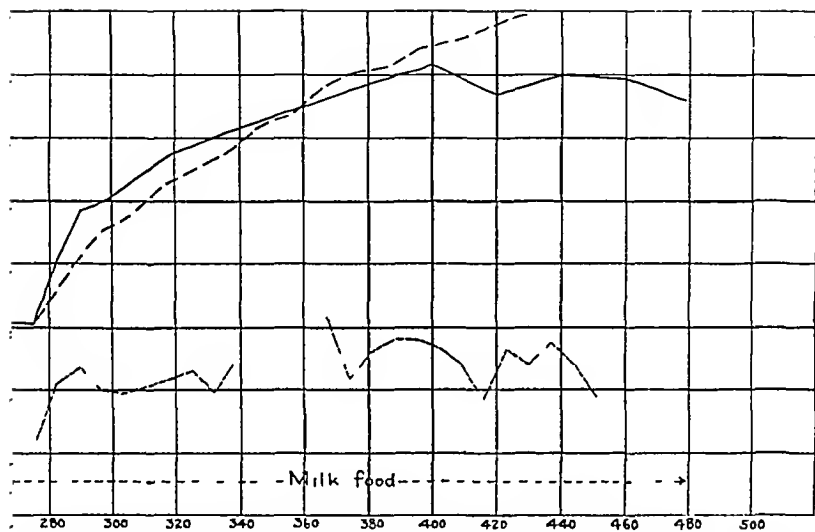


CHART 7, RAT 240 ♀, shows failure to make more than slight growth on a  $\frac{1}{2}$  rate after 276 days of stunting. At this time the rat was 314 days old, an  $\frac{1}{2}$  rate. The diet during periods 1 and 2 was

## PERIOD 1

Gliadin (wheat)  
 Protein free milk  
 Starch  
 Agar  
 Lard

per rat  
 110 Milk powder  
 25 starch  
 25 Lard  
 10  
 20  
 190



taining gliadin as the sole protein, and capacity to resume growth at a normal  
uch rats normally grow very little more

## PERIOD 2

	<i>per cent</i>
Milk powder	60 0
starch	16 0
lard	24 0
	<hr/>
	100 0

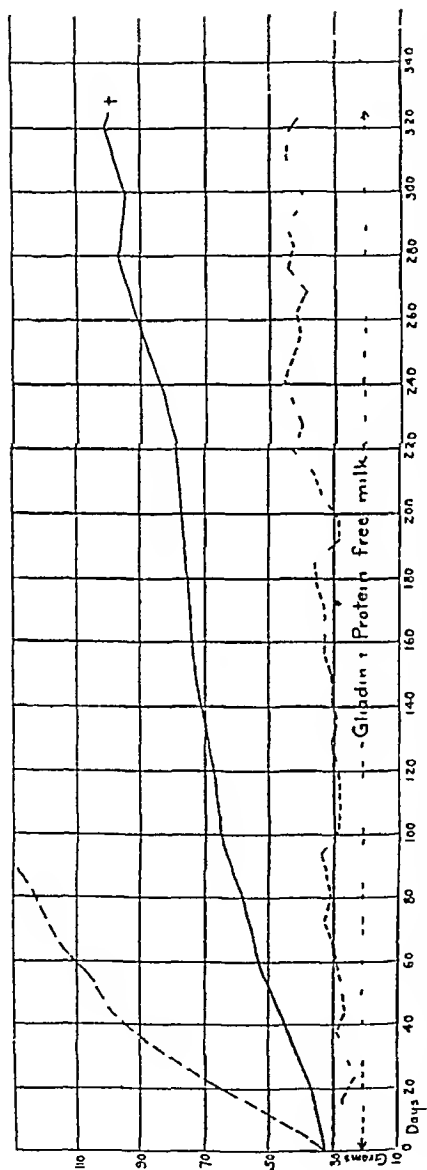


CHART 8, RAT 249 ♀, shows failure to make more than slight growth, during 325 days, on a food containing gliadin as its sole protein. This experiment was terminated by the death of the rat caused by sudden occlusion of the urethra with calculi.

The diet was

Gliadin (wheat)	per cent
Protein free milk	18 0
Starch	28 2
Agar	20 8
Lard	5 0
	28 0
	<hr/> 100 0

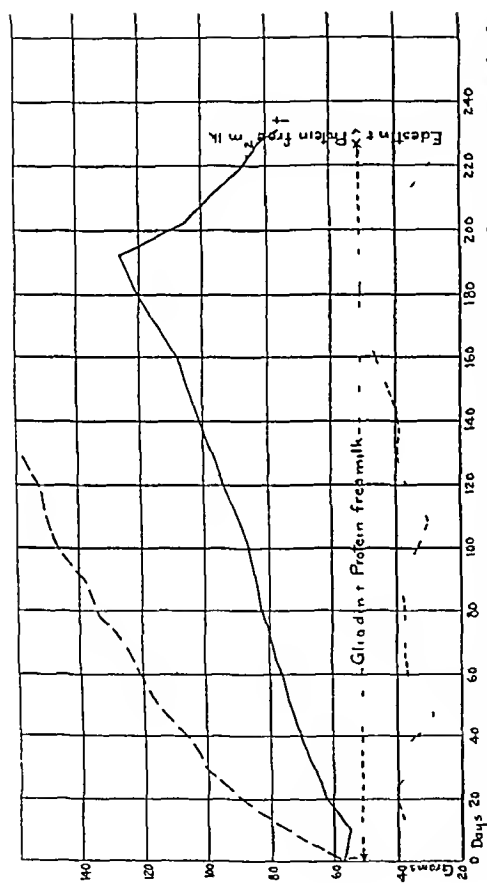


CHART 9, RAT 254 ♀, shows failure to grow normally on a diet containing gliadin as its sole protein. In this experiment growth was greater in extent and more rapid than in any of a large number of similar trials with this diet. The rat died after 231 days of experimental feeding with diseased lungs.

The diet was

Gliadin (wheat)  
Protein free milk  
Starch  
Agar  
Lard

per cent  
18 0  
28 2  
23 8  
5 0  
25 0  
100 0

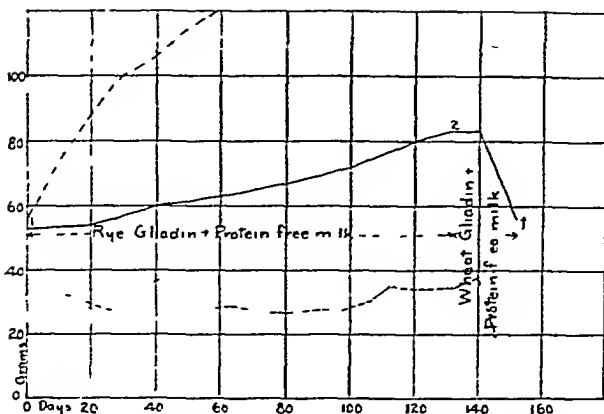


CHART 10, RAT 534 ♀, shows failure to make more than slight growth on a diet containing gliadin from rye as its sole protein. The animal died after 152 days of experimental feeding with diseased lungs.

The diet was

	PERIOD 1 per cent	PERIOD 2 per cent
Gliadin (rye)	18.0	0.0
Gliadin (wheat)	0.0	18.0
Protein-free milk	28.0	28.0
Starch	28.0	28.0
Lard	26.0	26.0
	100.0	100.0

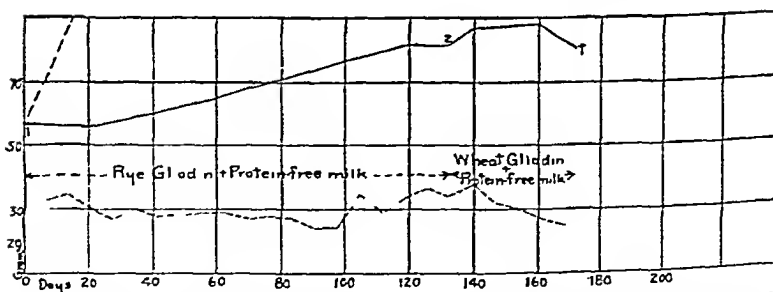


CHART 11, RAT 549 ♂, shows failure to make more than slight growth on diets containing gliadin from rye and later gliadin from wheat. This experiment was terminated by the death of the rat after 172 days of experimental feeding. The only abnormal condition revealed by the autopsy was a collection of hair balls in the stomach.

The diet during periods 1 and 2 was

	PERIOD 1 per cent	PERIOD 2 per cent
Gliadin (rye)	18.0	0.0
Gliadin (wheat)	0.0	18.0
Protein-free milk	28.0	28.0
Starch	28.0	26.0
Lard	26.0	28.0
	100.0	100.0

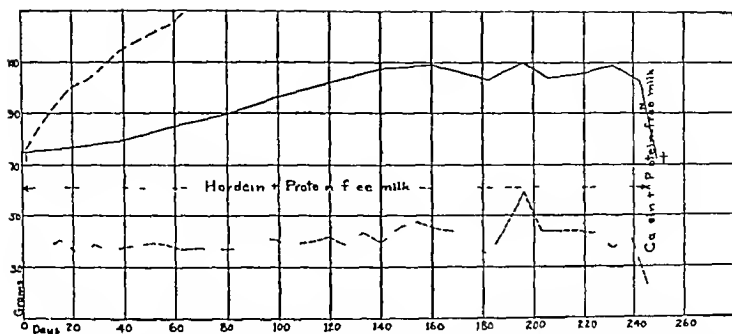


CHART 12, RAT 255 ♀, shows failure to make more than slight growth on a diet containing hordein from barley as the sole protein. Hordein is very much like gliadin in physical properties and amino acid make up and appears to have a similar value in nutrition. This rat died suddenly after 249 days of experimental feeding but no cause for death was shown by the autopsy.

The diet during periods 1 and 2 was

PERIOD 1			PERIOD 2		
	per cent			per cent	
Hordein	18.0	Casein		18.0	
Protein-free milk	28.2	Protein free milk		28.2	
Starch	18.8	Starch		18.8	
Agar	5.0	Agar		5.0	
Lard	30.0	Lard		30.0	
	100.0			100.0	

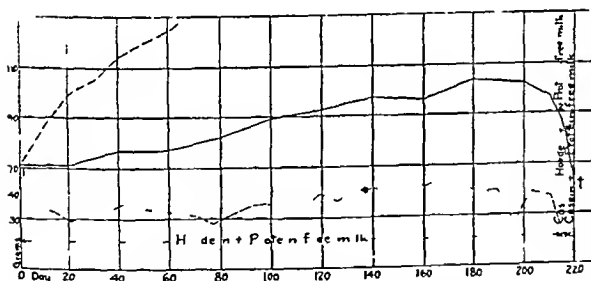


CHART 13, RAT 256 ♀ shows failure to make more than slight growth on a diet containing hordein from barley as the sole protein. Hordein is very much like gliadin in physical properties and amino acid make-up and appears to have a similar value in nutrition. The animal died suddenly after 220 days of experimental feeding but an autopsy failed to show anything abnormal.

The diet during periods 1 and 2 was

PERIOD 1			PERIOD 2		
	per cent			per cent	
Hordein	18.0	Casein		18.0	
Protein free milk	28.2	Protein free milk		28.2	
Starch	18.8	Starch		18.8	
Agar	5.0	Agar		5.0	
Lard	30.0	Lard		30.0	
	100.0			100.0	





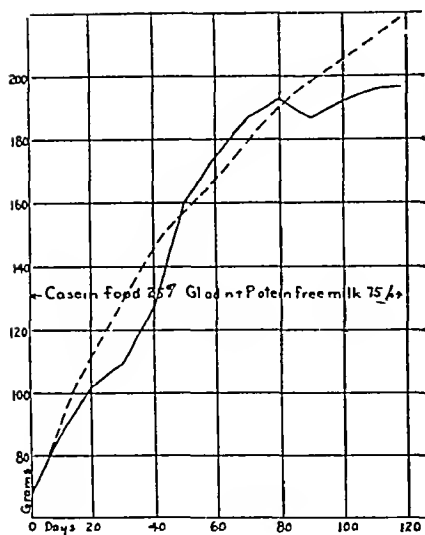


CHART 16, RAT 287 ♂, shows normal growth on a diet in which the protein consisted of 1 part casein and 3 parts of gliadin. Note the effect on the rate of growth induced by this small proportion of casein. Cf Charts 7, 8, 10, 13, 18 and 19.

The diet consisted of a mixture of one part of the casein food with three parts of the gliadin food.

## GLIADIN FOOD

	per cent
Gliadin	18 0
Protein free milk	28 2
Starch	28 8
Agar	5 0
Lard	28 0
	<hr/> 100 0

## CASEIN FOOD

	per cent
Casein	18 0
Starch	32 5
Sucrose	17 0
Agar	5 0
Salt mixture I	2 5
Lard	25 0
	<hr/> 100 0

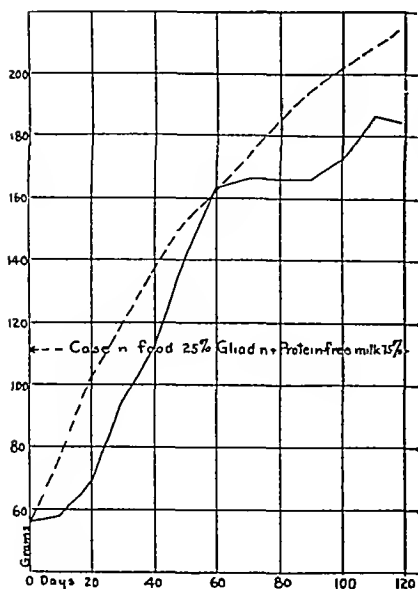


CHART 17, RAT 280 ♂, shows normal growth on a diet in which the protein consisted of 1 part casein and 3 parts of gliadin. Note the effect on the rate of growth induced by this small proportion of casein. Cf Charts 7, 8, 10, 13, 18 and 19.

The diet consisted of a mixture of one part of the casein food with three parts of the gliadin food.

## GLIADIN FOOD

	per cent
Gliadin	18 0
Protein free milk	28 2
Starch	28 8
Agar	5 0
Lard	28 0
	<hr/> 100 0

## CASEIN FOOD

	per cent
Casein	18 0
Starch	32 5
Sucrose	17 0
Agar	5 0
Salt mixture I	2 5
Lard	25 0
	<hr/> 100 0

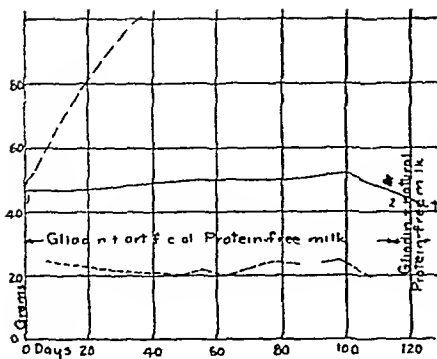


CHART 18, RAT 588 ♀, shows failure to grow on a diet containing gliadin as the sole protein and an artificial imitation of the natural protein-free milk. After 114 days the artificial protein-free milk was replaced by natural, but the decline in weight which had begun was not stopped by this change. The autopsy showed no adequate cause for death. The diet in periods 1 and 2 was

	per cent		per cent
Gliadin	18 0	Gliadin	18 0
Artificial protein free milk	30 0	Protein free milk	28 0
Starch	22 0	Starch	26 0
Lard	30 0	Lard	28 0
	100 0		100 0

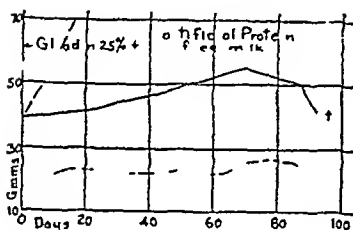


CHART 19, RAT 594 ♂, shows failure to make more than slight growth on a diet containing gliadin as its sole protein and an artificial imitation of the natural protein-free milk. The animal died after 92 days of experimental feeding. Calculi were found in the bladder and left kidney.

The diet was

	per cent
Gliadin	25 0
Artificial protein free milk	30 0
Starch	15 0
Lard	30 0
	100 0

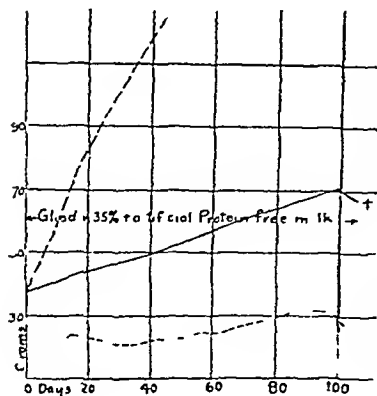


CHART 20, RAT 603 ♂, shows failure to grow at normal rate on a diet containing gliadin as the sole protein. In this experiment an artificial imitation of the natural protein-free milk was used. This animal died after 105 days of experimental feeding with diseased lungs.

The diet was

	per cent
Gliadin	35 0
Artificial protein free milk	30 0
Starch	5 0
Lard	30 0
	100 0

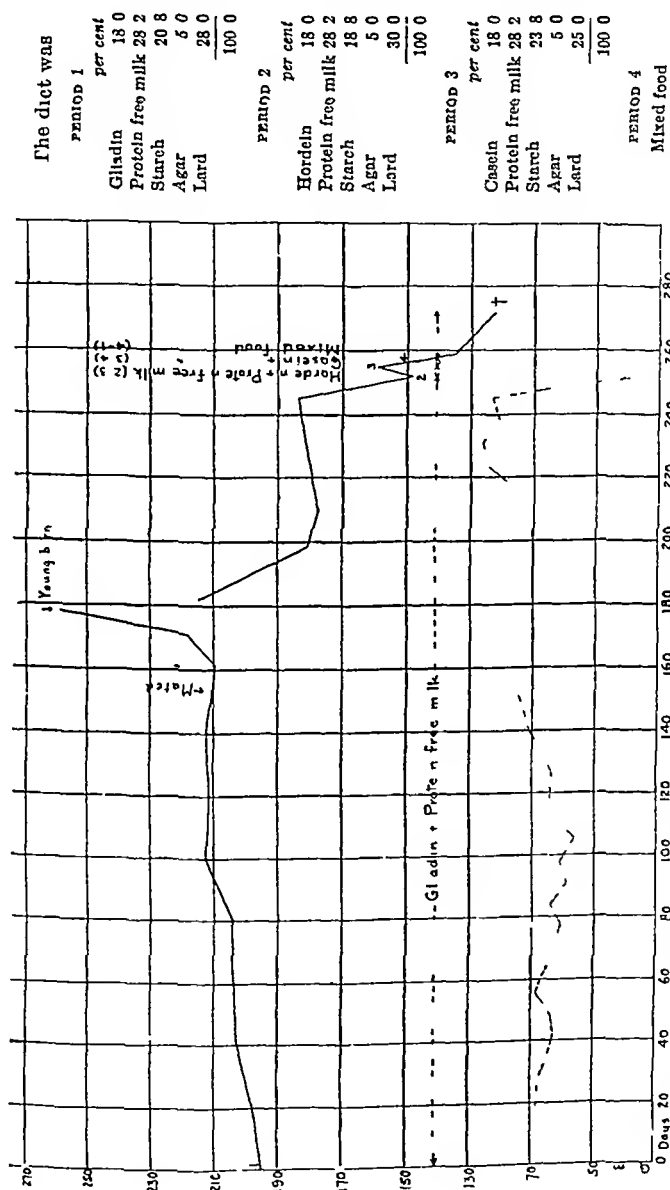


CHART 21, Rat 129 ♀, shows maintenance and production of young after feeding for 178 days on a diet containing gliadin as its sole protein. The rat died after 272 days of experimental feeding apparently from some disease since neither a change in protein or even mixed diet stopped the decline. Unfortunately no autopsy was made. Note the very great and rapid gain in weight during pregnancy in spite of the fact that the food contained gliadin as the sole protein.

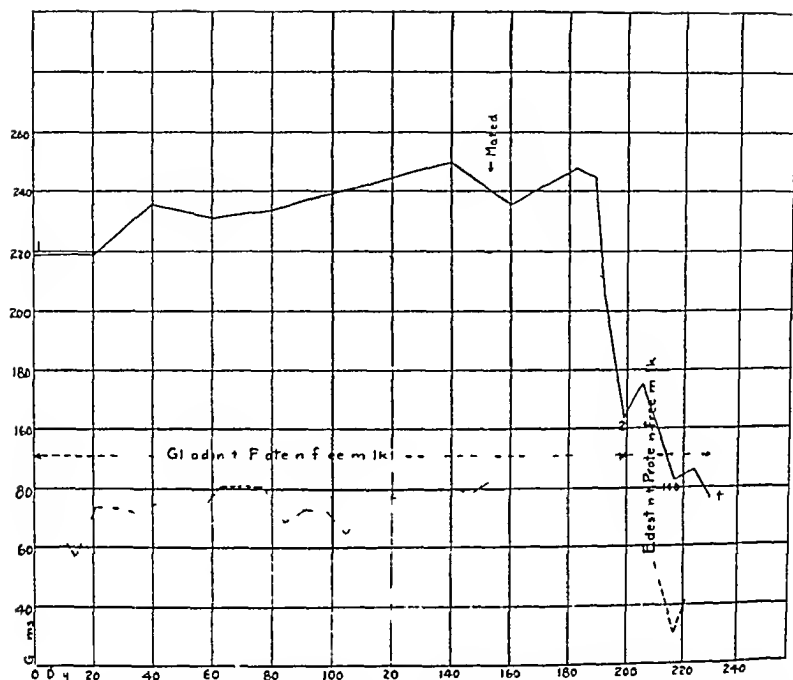


CHART 22, RAT 168 ♂, shows maintenance and fertility on a diet containing ghadin as its sole protein. After 154 days this rat was paired with Rat 129, four young being the result of the mating. The animal died with diseased lungs after 230 days of experimental feeding.

The diet was

PERIOD 1		PERIOD 2	
	per cent		per cent
Ghadin	18.0	Edestin	18.0
Protein-free milk	28.2	Protein free milk	28.2
Starch	20.8	Starch	20.8
Agar	5.0	Agar	5.0
Lard	28.0	Lard	28.0
	100.0		100.0

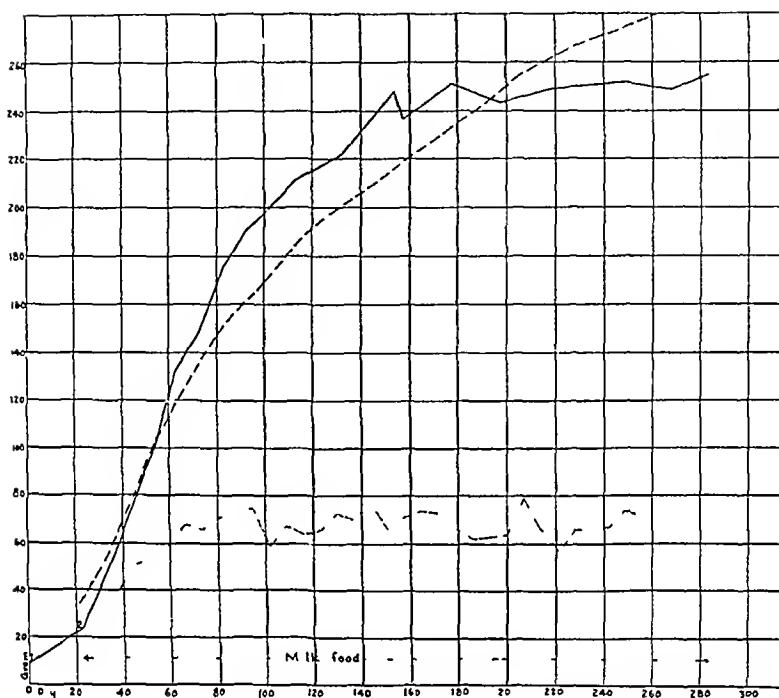


CHART 23 RAT 378 ♂ shows normal growth during 284 days on a diet containing all of the ingredients of milk. Note the vigorous growth of this animal which was produced and suckled period 1 by a mother whose food during the previous 178 days contained gliadin as its sole protein.

The diet was

PERIOD 2

Milk powder	per cent
Starch	60.0
Lard	16.0
	<u>24.0</u>
	100.0

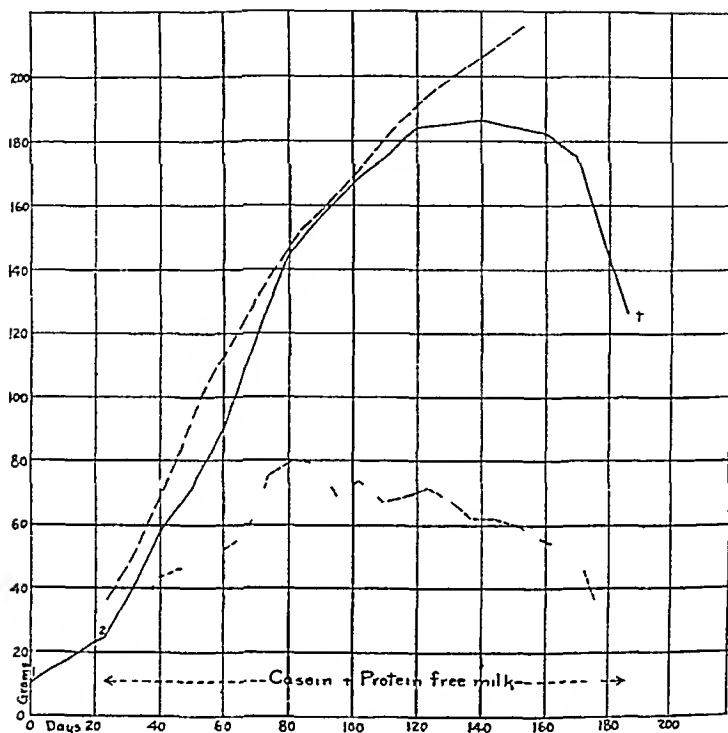


CHART 24, RAT 379 ♂, shows normal growth on a diet containing casein as its sole protein up to an age of 120 days. The subsequent fall in weight is characteristic for animals thus fed and will be discussed in a later paper. This animal was produced and suckled by a mother previously fed for 178 days on a diet containing gliadin as its sole protein.

The food during period 1 was its mother's milk, during period 2 as follows

#### PERIOD 2

	per cent
Casein	18.0
Protein-free milk	28.2
Starch	23.8
Agar	5.0
Lard	25.0
	<hr/> 100.0

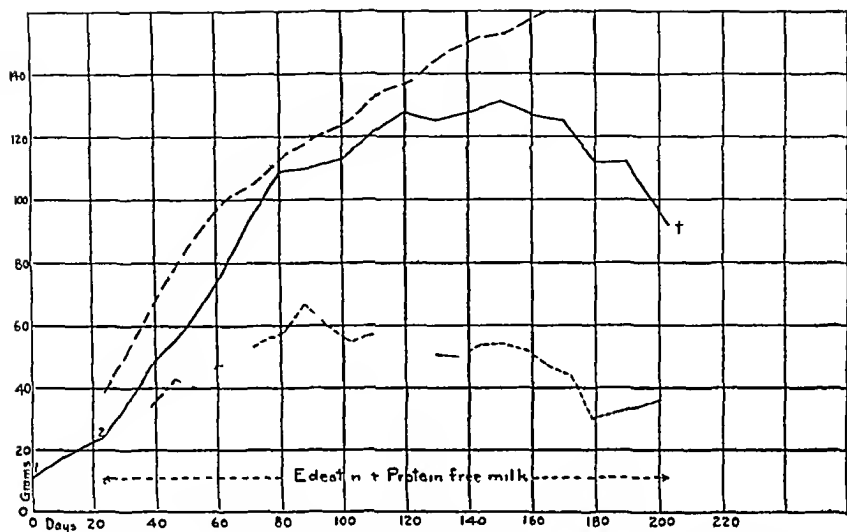


CHART 25, RAT 380 ♀, shows normal growth on a diet containing edestin as its sole protein up to an age of 120 days. The subsequent fall in weight is characteristic for animals thus fed and will be discussed in a later paper. This animal was produced and suckled by a mother previously fed for 178 days on a diet containing gliadin as its sole protein.

The food during period 1 was its mother's milk, during period 2 as follows

## PERIOD 2

	per cent
Edestin	18.0
Protein-free milk	28.2
Starch	20.8
Agar	5.0
Lard	28.0
	<hr/> 100.0



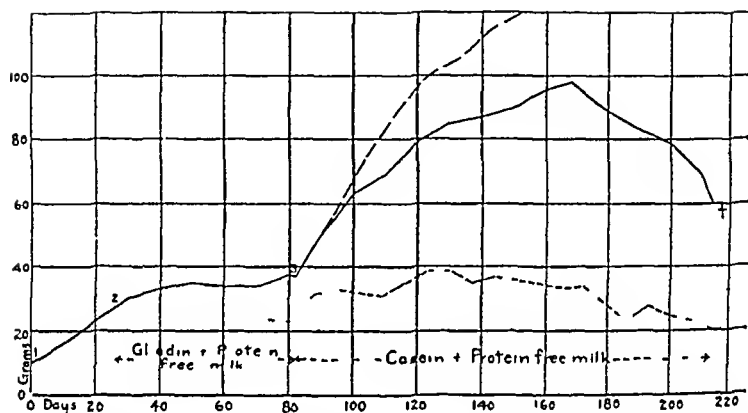


CHART 26, RAT 381 ♀, shows failure to grow on a diet containing gladin as its sole protein, period 2, and resumption of growth when the gladin of the food was replaced by casein, period 3. The experiment was terminated by the death of the animal. Autopsy showed calculi in the kidneys, ureters and bladder. This animal was produced by a mother previously fed for 178 days on a diet containing gladin as its sole protein. During period 1 it was suckled by its mother.

The diet was

PERIOD 2		PERIOD 3	
	per cent		per cent
Gladin	18.0	Casein	18.0
Protein free milk	28.2	Protein free milk	28.2
Starch	20.8	Starch	27.0
Agar	5.0	Lard	27.0
Lard	28.0		100.0
	100.0		

# THE CHEMISTRY OF GLUCONEOGENESIS

## I THE QUANTITATIVE CONVERSION OF PROPIONIC ACID INTO GLUCOSE

B. A. I. RINGER

(From the Department of Physiological Chemistry of the University of Pennsylvania)

(Received for publication, August 2, 1912)

Within the last ten years it has been shown conclusively that in diabetes the glucose may find its origin in protein. Stiles and Lusk<sup>1</sup> gave a diabetic dog a mixture of amino-acids which had been obtained from a pancreatic digest of meat. They found an increase in the sugar elimination corresponding to about 40 per cent of the administered amino-acids. Since then, several investigators have studied the fate of the individual amino-acids in the diabetic organism.

Knopf<sup>2</sup> obtained a decided increase in the glucose elimination in a phlorhizinized dog that was fed on meat and to whose diet 50 grams of asparagine had been added. Embden and his confrères<sup>3</sup> fed glycocoll and alanine to depancreatized dogs and obtained a considerable increase in the glucose elimination. Ringer and Lusk<sup>4</sup> working on phlorhizinized dogs, showed that glycocoll and  $\alpha$ -alanine may be completely converted into glucose, and that aspartic and  $d$ -glutamic acid may yield glucose corresponding to about three carbon atoms of their respective molecules. Mandel and Lusk<sup>5</sup> showed that lactic acid may also be completely converted into glucose. Hockendorf<sup>6</sup> showed that the feeding of propyl

<sup>1</sup> *Amer Journ of Physiol*, 14, p 380, 1903

<sup>2</sup> *Arch f exp Path u Pharm*, xlix, p 123, 1903

<sup>3</sup> *Beitr z chem Physiol u Path*, v, p 507, 1904, vii, p 298, 1906

<sup>4</sup> *Zeitschr f physiol Chem*, lxxvi, p 106, 1910

<sup>5</sup> *Amer Journ of Physiol*, xvi, p 129, 1906

<sup>6</sup> *Biochem Zeitschr*, xliii, p 281, 1909

## 512 Conversion of Propionic Acid into Glucose

alcohol may give rise to glucose This last finding was corroborated by Ringer and Lusk <sup>7</sup>

In the present series of investigations, it is the author's object to find which chemical radicals determine the conversion or non-conversion of amino-acids and chemically related substances into glucose In this paper, the fate of propionic acid in diabetic dogs will be discussed

*Methods* The methods employed in this series of researches are similar to those described by Ringer and Lusk and need not be repeated here Merck's phlorhizin was used and the propionic acid was prepared by Kahlbaum

The results are as follows

### EXPERIMENT I

#### Twelve-hour periods

DATE May 1912	PERIOD	WEIGHT	TOTAL N	TOTAL GLUCOSE	D N	NH <sub>3</sub> N	ACETONE AND ACETO-ACETIC ACID	$\beta$ -oxy-butyric ACID	REMARKS
20	IV	kilos 12 9	grams 6 5	grams 22 5	3 46	gram 0 39	gram 0 30	grams 2 07	Fasting
20	V	12 4	6 2	30 7	5 00	0 34	0 19	2 16	10 grams of propionic acid (neutralized with NaOH) given subcutaneously in two doses
21	VI		5 5	23 9	4 35	0 38	0 43	2 71	Fasting

This animal was in good shape throughout the experiment The first three periods were devoted to other studies, which will be reported elsewhere At the beginning of the fifth period, the animal received subcutaneously 10 grams of propionic acid which had been dissolved in 70 cc of water and neutralized with NaOH As is seen from the D N ratio, not all of the extra glucose was eliminated in that period, but some was carried over to the sixth

<sup>7</sup> *Loc cit*

period<sup>8</sup> The D N ratio fluctuates from day to day to but a slight extent Any change that may occur is usually in a downward direction, and this method of calculating the "extra" glucose really represents minimal results If we assume that, without the influence of the propionic acid, the D N ratio in periods V and VI would have continued at 3.46:1 (since the animal eliminated 11.7 grams of nitrogen in these two periods) then the amount of glucose in these two periods should have been  $(11.7 \times 3.46)$  40.5 grams The actual amount of glucose eliminated was 54.6 grams, which shows that 14.1 grams of glucose came from the 10 grams of propionic acid

## EXPERIMENT II

## Twelve-hour periods

DATE June 1912	PERIOD	WEIGHT	TOTAL N	TOTAL GLUCOSE	D N	NH <sub>3</sub> -N	ACETONE AND ACETO-ACETIC ACID	$\beta$ -OXY BUTYRIC ACID	REMARKS
15	IV	kilos 14.0	grams 5.4	grams 19.3	3.58	gram 0.37	gram 0.23	grams 1.94	Fasting -
15	V		4.6	24.9	5.46	0.18	0.16	1.59	10 grams of propionic acid as above given per os
16	VI		4.4	19.1	4.33	0.18	0.19	2.29	Fasting
16	VII		4.5	15.1	3.37	0.23	0.13	2.31	Fasting

In this experiment, as in the first, "extra" glucose was eliminated after feeding sodium propionate The D N ratio in the fore and after period was 3.58 and 3.37, if we take the mean—3.47—for periods V and VI, and apply the same method of calculation as above, we find that the 10 grams of propionic acid yielded in this case 12.8 grams of glucose

Experiment III is corroborative of the first two Here the "extra" glucose is completely eliminated in period III The D N ratio in periods II and IV is 3.9 and 3.65 By taking the mean of

<sup>8</sup> The extra glucose was identified as such through the correspondence of the figures obtained by the polariscopic determination with those of Allihn's gravimetric method

EXPERIMENT III  
Twelve-hour periods

DATE June 1912	PERIOD	WEIGHT	TOTAL N	TOTAL GLUCOSE	D N	ACETONE AND ACETO-ACETIC ACID	REMARKS
2	II	kilos 12 3	grams 8 20	grams 31 97	3 90	gram 0 212	Fasting
2	III		8 06	47 46	5 88	0 070	10 grams of propionic acid neutralized with $(\text{NH}_4)_2\text{CO}_3$ given subcutaneously in two doses
3	IV		8 30	30 30	3 65	0 192	Fasting
3	V	11 71	8 32	28 29	3 40	0 196	Fasting
4	VI		8 08	29 34	3 63	0 222	Fasting

these two as the ratio for period III, we find that  $17.1 (8.06 \times 3.77 = 30.39 - 47.46 - 30.39 = 17.07)$  grams of glucose came from the propionic acid

DISCUSSION

If all of the carbon of the propionic acid goes over into glucose, 10 grams of propionic acid can give rise to 12.2 grams of glucose



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In the experiments reported above, we find that in every case more glucose was eliminated than can be theoretically attributed to the propionic acid

EXPERIMENT	EXTRA GLUCOSE	GRAMS ABOVE THEORETICAL GLUCOSE VALUE OF PROPIONIC ACID
I	14 1	1 9
II	12 8	0 6
III	17 1	4 9

The excess in the first two experiments may be attributed to errors in calculating the "assumed" D/N ratio. The value in III is a little too high. Its significance will be investigated in the near future.

Up to the present time, it was believed that for the conversion of a substance into glucose, an alcohol, aldehyde or a ketone radical in the molecule was essential. This is the first proof to the contrary. It shows very clearly that a fatty acid may be converted into glucose.

#### SUMMARY

Three experiments were performed on phlorhizinized dogs, which received 10 grams of propionic acid either subcutaneously or *per os*. The results justify the conclusion that the propionic acid is completely converted into glucose.



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